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(54) Title: MARKER ASSISTED SELECTION OF BOVINE FOR IMPROVED MILK PRODUCTION USING DIACYLGLYCEROL ACYLTRANSFERASE GENE DGAT1

(57) Abstract: The present invention provides a method of genotyping bovine for improved milk production traits by determining the DGAT1 genotypic state of said bovine, wherein the DGAT1 gene and polymorphisms within said gene have been found to be associated with such improved milk production traits.

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**Marker Assisted Selection of Bovine for Improved Milk Production Using Diacylglycerol
Acyltransferase gene DGAT1**

FIELD OF THE INVENTION

5 This invention relates to an application of marker assisted selection of bovine for a
quantitative trait loci (QTL) associated with milk production, particularly although by
no means exclusively, by assaying for the presence of at least one allele which is
associated with increased milk volume as well as improved milk composition. The
present invention also relates to the gene associated with the QTL, various
10 polymorphisms within the gene sequence, proteins encoded by these sequences as well
as to the application of all of these in the farming industry.

BACKGROUND

15 The genetic basis of bovine milk production is of immense significance to the dairy
industry. An ability to modulate milk volumes and content has the potential to alter
farming practices and to produce products which are tailored to meet a range of
requirements. In particular, a method of genetically evaluating bovine to select those
which express desirable traits, such as increased milk production and improved milk
20 composition, would be desirable.

To date, bovine genomics are poorly understood and little is known regarding the genes
which are critical to milk production. While there have been reports of quantitative trait
loci (QTLs) on bovine chromosome 14 postulated to be associated with milk production
25 (Coppieters *et al* (1998)), the specific genes involved have not to date been identified.

Marker assisted selection, which provides the ability to follow a specific favourable
genetic allele, involves the identification of a DNA molecular marker or markers that
segregate with a gene or group of genes associated with a QTL. DNA markers have
30 several advantages. They are relatively easy to measure and are unambiguous, and as
DNA markers are co-dominant, heterozygous and homozygous animals can be
distinctively identified. Once a marker system is established, selection decisions are
able to be made very easily as DNA markers can be assayed at any time after a DNA
containing sample has been collected from an individual infant or adult animal, or even
35 earlier as it is possible to test embryos in vitro if such embryos are collected.

The applicants have now identified a gene responsible for the QTL effect on bovine chromosome 14 as well as a number of polymorphisms which are associated with distinct genetic merits of animals for milk composition and volume.

- 5 It is an object of the present invention to provide an application method for marker assisted selection of this bovine gene, and in particular, of the polymorphisms in the bovine gene which are associated with increased milk volume and altered milk composition; and/or to provide genetic markers for use in such a method; and/or to provide the nucleic acid and amino acid sequences of this gene and encoded
10 polypeptide; and/or to provide animals selected using the method of the invention as well as milk produced by the selected animals; and/or to provide the public with a useful choice.

SUMMARY OF THE INVENTION

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This invention relates to the discovery of the bovine Diacylglycerol-o-acyltransferase (DGAT1) gene and polymorphisms within the bovine DGAT1 gene which are associated with increased milk yield and altered milk composition.

- 20 More specifically, several polymorphisms in the bovine DGAT1 gene have been identified distinguishing multiple DGAT1 alleles in different cattle breeds. These polymorphisms include: K232A (Bases 6829/30 AA-CG nucleic acid change and K-A amino acid change); Nt984+8(Base 7438 A-G nucleic acid change); Nt984+26(Base 7456 C-T nucleic acid change); Nt1470+85(Base 8402 C-T nucleic acid change); Nt191+435 (Base
25 626 T-G nucleic acid change); Nt191-3321 (Base 3512 T-G nucleic acid change); Nt279+144 (Base 4040 T-C nucleic acid change); Nt279+1067 (Base 4963 A-G nucleic acid change); Nt279+1107 (Base 5003 G-A nucleic acid change); Nt358 (Base 5997 C-T nucleic acid change); Nt754+3 (Base 6892 G-A nucleic acid change); Nt897+32 (Base 7224/5 GG-AC nucleic acid change); Nt1251+42 (Base 7987 G-A nucleic acid change)
30 as summarised in Table 1. In particular, DGAT1 alleles characterized by the K232A mutation have been identified as being associated with an increased milk volume and altered milk composition in animals dependent upon whether they are homozygous with or without the mutation or heterozygous carrying one mutated allele. More specifically, the presence of the K232A mutation results in a decrease in milkfat percentage, milkfat
35 yield, solid fat content and milk protein percentage, while increasing milk volume and milk protein yield.

The present invention thus relates to the use of the polymorphisms in a method of identification and selection of a bovine having at least one of said polymorphisms as well as to providing markers specific for such identification. Kits comprising said markers for use in marker selection also form part of the present invention as do
5 animals so selected, the milk produced by such selected animals and products produced from such milk, particularly as such milk and milk products affect processing and/or health characteristics for consumers.

In particular, the present invention is directed to a method of genotyping cows or bulls
10 for one or more of the polymorphisms disclosed herein, selected cows or bulls so genotyped and milk and semen from said selected cows and bulls respectively.

According to a further aspect the present invention is directed to the isolated DGAT1 nucleic acid and allelic nucleic acid molecules comprising polymorphisms as well as to
15 the proteins encoded thereby and their polypeptide sequences. Antibodies raised against said proteins are also contemplated, as are vectors comprising the nucleic acid molecules, host cells comprising the vectors; and protein molecules expressed in said host cells; and the application of all of them in the farming industry.

In particular, such applications include methods for modulating milk production and/or composition in a lactating bovine by affecting DGAT1 activity, by reducing the activity of DGAT1 (e.g. by use of specific ribozymes, antisense sequences and/or antibodies, or by transgenic technology to produce a "knock out" bovine and/or bovine with introduced transgenes containing the DGAT1 gene and/or variations of this gene
25 driven by various promoters).

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described with reference to the Figures of the accompanying
30 drawings in which:

Figure 1: Shows a BAC contig spanning the *BULGE13-BULGE09* interval relative to a schematic diagram of bovine chromosome 14 and a schematic diagram showing the location of the genetic markers. The most likely position of the QTL is shown as a bar on the FISH-anchored linkage map proximal to BTA14q. The BACs composing the
35 contigs spanning the *BULGE13-BULGE09* interval are shown as a series of horizontal lines. The symbols on each BAC indicate their individual STS content: solid circles

correspond to STS derived from BAC ends, open boxes to microsatellite markers, and solid triangles to gene-specific Comparative Anchored Tagged Sequences. The arrow heads mark the BACs from which the respective BAC end STS were derived. The length of the lines do not reflect the actual insert size of the corresponding BACs. The BAC contig was aligned with the orthologous human HSA8q24.3 genomic "golden path" sequence represented according to the Ensembl Human Genome Server (<http://www.ensembl.org/>): individual sequence contigs are shown in alternating light and dark; a horizontal line indicates a gap in the sequence assembly; genetic markers are indicated under the contig map; the lines and boxes above the contig map represent "curated", "predicted known" or "predicted novel" genes.

Figures 2a and 2b: Show the genomic sequence of the bovine DGAT1 gene. Figure 2a is the 31 base pair sequence upstream but adjacent to the ATG or translation start site and is 5'UTR. Figure 2b is the genomic sequence in the bovine DGAT1 gene from the ATG translation start site (base 1) through to genomic sequence flanking the gene at the 3' end. The significant features including intron/exon boundaries, polymorphic sites, polyadenylation signal, and alternate splicing site and some of the primer sequences used in the assays described herein, are indicated;

Figure 3: Shows the genomic organization, four polymorphisms and haplotypes found in the bovine DGAT1 gene. Leader and trailer sequences are shown in light grey, coding sequences in dark grey and intronic sequences as a hollow line. The positions of four of the identified polymorphisms are marked as shown on the gene, and detailed in the underlying boxes including the corresponding sequence traces All the sequence variations are summarised in Table 1. The four DGAT1 haplotypes which were found in the Dutch and New-Zealand Holstein-Friesian population as defined by these polymorphisms are shown and referred to as "sHQ-D", "sHQ-NZ", "sHQ-III" for the fat increasing haplotypes and "shs" for the fat decreasing haplotype;

Figure 4a: Shows the corresponding full length amino acid sequence for DGAT1 sequence of Figure 2b including annotation of the amino acid substitution;

Figure 4b: Shows the amino acid sequence predicted as a result of alternate splicing with exon VIII;

Figure 5: Shows the multiple peptide alignment of a portion of the DGAT1 protein flanking the K232A substitution from *Bos taurus*, *Bison bison*, *Ovis aries*, *Sus scrofa*,

Homo sapiens, *Cercopithecus aethiops*, *Mus musculus domesticus* and *Rattus norvegicus* showing the evolutionary conservation of the lysine mutated in the bovine K232A polymorphism;

- 5 **Figure 6:** A. Shows the frequency distribution of observed *DGAT1* SNP haplotypes in the Dutch and New Zealand Holstein-Friesian dairy cattle populations. B-D. Shows the frequency distribution of the combined microsatellite (*BULGE09-BULGE11*) and SNP *DGAT1* haplotypes. The *HQ-D* and *HQ-NZ* haplotypes are shown; and
- 10 **Figure 7:** Shows the lod score due to LD when including (+) or excluding (-) the four *DGAT1* polymorphisms shown in Figure 3 in a combined linkage and LD multipoint maximum likelihood mapping method. The lod score corresponds to the \log_{10} of the ratio between the likelihood of the data assuming LD and linkage between the markers and the likelihood of the data assuming linkage in the absence of LD. The positions of
- 15 the microsatellites and SNP markers utilized in the analysis are shown on the X-axis, while the position of the *DGAT1* SNPs is marked by a red arrow at the top of the figure.

DETAILED DESCRIPTION OF THE INVENTION

- 20 It has been discovered for the first time that the *DGAT1* gene in bovine is associated with the QTL on chromosome 14 which is linked with improved milk production traits. More particularly, a number of novel polymorphisms on the *DGAT1* gene have been discovered. It is thought that one or more of these polymorphisms is responsible for these traits.

- 25 The method used for isolating genes which cause specific phenotypes is known as positional candidate cloning. It involves: (i) the chromosomal localisation of the gene which causes the specific phenotype using genetic markers in a linkage analysis; and (ii) the identification of the gene which causes the specific phenotype amongst the
- 30 "candidate" genes known to be located in the corresponding region. Most of the time these candidate genes are selected from available mapping information in humans and mice.

- The tools required to perform the initial localisation (step (i) above) are microsatellite marker maps, which are available for livestock species and are found in the public domain (Bishop *et al.*, 1994; Barendse *et al.*, 1994; Georges *et al.*, 1995; and Kappes, 1997). The tools required for the positional candidate cloning, particularly the BAC libraries, (step (ii) above) are partially available from the public domain. Genomic
- 35

libraries with large inserts constructed with Bacterial Artificial Chromosomes (BAC) are available in the public domain for most livestock species including cattle. For general principles of positional candidate cloning, see Collins, 1995 and Georges and Anderson, 1996.

5

Recently, a quantitative trait locus (QTL) with major effect on milk solids composition, located at the centromeric end of bovine chromosome 14, has been reported (Coppieters *et al.*, (1998)). This QTL was shown to effect milk fat content and in particular to significantly affect protein %, volume, protein yield and fat yield of milk. The linkage study as well as subsequent marker assisted segregation analyses allowed for the identification of thirteen Holstein-Friesian sires predicted to be heterozygous "Qq" for the corresponding QTL (Coppieters *et al.*, (1998); Riquet *et al.*, (1999)).

10

Linkage disequilibrium methods were applied to refine the map position of the QTL to a ≈ 5 cM interval bounded by microsatellite markers BULGE09 and BULGE30.

15

A bovine DGAT1 nucleotide sequence was determined by the applicants and is shown in Figures 2a and 2b with the corresponding amino acid sequences (long and short forms) being shown in Figures 4a and 4b respectively. Table 1 sets out all the polymorphisms located to date with reference to the sequence in Figure 2b. Some of the genetic polymorphisms identified in the bovine DGAT1 gene are reported in Figure 3. The nucleic acid and protein sequences of the DGAT1 alleles including the K232A mutation are shown in Figures 2a and 2b (SEQ ID NOs: 3 and 1), annotated to show the alternatively spliced forms. The cDNA sequence is also set out in SEQ ID NO: 4.

20

25

The sequence information in the Figures gives rise to numerous, and separate, aspects of the invention.

30

In one aspect, the invention provides a method of determining genetic merit of a bovine with respect to milk composition and volume which comprises the step of determining the bovine DGAT1 genotypic state of said bovine. In particular, this method is useful for genotyping and selecting cows and bulls having the desired genotypic state so that milk and semen may be collected from said cows and bulls respectively. Such semen would be useful for breeding purposes to produce bovine having the desired genotypic and, as a result, phenotypic state. In addition, cows genotyped by the methods of the present invention are also useful for breeding purposes, particularly for breeding with the selected bulls and/or to be artificially inseminated with the semen from selected

35

bulls. The embryos and offspring produced by such cows also form part of the present invention.

5 In one embodiment, the genotypic state is determined with respect to DNA obtained from said bovine.

Alternatively, said genotypic state is determined with reference to mRNA obtained from said bovine.

10 In yet a further embodiment, the genotypic state is determined with reference to the amino acid sequence of expressed bovine DGAT1 protein obtained from said bovine.

Conveniently, in said method, the genotypic state of DNA encoding bovine DGAT1 is determined, directly or indirectly.

15 Alternatively, in said method the genotypic state of at least one nucleotide difference from the nucleotide sequence encoding bovine DGAT1 is determined, directly or indirectly.

20 More specifically, in said method the genotypic state of bovine DGAT1 allele(s) characterised by one or more of the polymorphisms shown in Table 1 below, is determined, directly or indirectly.

Table 1:
Table of polymorphisms in the bovine DGAT1 gene

5 Start codon (atg); the a residue is denoted as position 1

Base number relative to exonic sequence ¹	Nucleotide from start	distance	substitution	Intron/exon #
Nt 191 + 435	626		T-G CAGTGCTAGGGG CAGTGCGAGGGG	Intron 1
Nt 191 + 3321	3512		T-G GCATTGCGCT GCATGGCGCT	Intron 1
Nt 279 + 144	4040		T-C TACCCTGGGAC TACCCGGGAC	Intron 2
Nt 279 + 1067	4963		A-G CTCTTAGCAGC CTCTTGGCAGC	Intron 2
Nt 279 + 1107	5003		G-A ACAGGCAACT ACAGACAAC	Intron 2
Nt 358	5997		C-T TGTCTCTGTTC TGTCTTTGTTC	Exon IV
Nt 692	6829		AA-GC GGTAAGAAGGCCAA (Q) GGTAAGGCGGCCAA (q)	K232A Exon VIII *
Nt 754 + 3	6892		G-A GCGGTGAGGAT GCGGTAAGGAT	Intron VIII
Nt 897 + 32	7224		GG-AC GGGGGGGGGGGACTCT GGGGGACGGGGACTCT	Intron X
Nt 984 + 8	7438		A-G GAGTGACCTGC GAGTGCCTGC	Intron XII *
Nt 984 + 26	7456		C-T GGACGCGTGGG GGACGTGTGGG	Intron XII *
Nt1251 + 42	7987		G-A GGTGGGGGTGG GGTGGAGGTGG	Intron XV
Nt 1470 + 85	8402		C-T CTGGGCGCAGC CTGGGTGCAGC	3' flanking region *

The numbers given are for the actual nucleotide or in the case of two nucleotide substitutions to the first nucleotide in the variation (counting 5' to 3')

10 *More detail of these polymorphisms is given in Figure 2b.

¹ e.g. Nt 191 represents nucleotide number 191 from the start site of the coding sequence, + 435 represents number of nucleotides from and including base 192 in the genomic sequence (intron 1) to the polymorphic nucleotide. The polymorphic nucleotides are shaded

15 Preferably, the invention is directed to a method of determining the genotypic state of bovine DGAT1 allele(s) by determining the presence of the K232A polymorphism, either directly or indirectly.

20 There are numerous art standard methods known for determining whether a particular DNA sequence is present in a sample. An example is the Polymerase Chain Reaction

(PCR). A preferred aspect of the invention thus includes a step in which ascertaining whether a polymorphism(s) in the sequence of DGAT1 DNA is present, includes amplifying the DNA in the presence of primers based on the nucleotide sequence of the DGAT1 gene and flanking sequence, and/or in the presence of a primer containing at least a portion of a polymorphism as disclosed herein and which when present results in altered relative milk lipid and protein production, and milk volume.

A primer of the present invention, used in PCR for example, is a nucleic acid molecule sufficiently complementary to the sequence on which it is based and of sufficient length to selectively hybridise to the corresponding portion of a nucleic acid molecule intended to be amplified and to prime synthesis thereof under *in vitro* conditions commonly used in PCR. Likewise, a probe of the present invention, is a molecule, for example a nucleic acid molecule of sufficient length and sufficiently complementary to the nucleic acid molecule of interest, which selectively binds under high or low stringency conditions with the nucleic acid sequence of interest for detection thereof in the presence of nucleic acid molecules having differing sequences.

In another aspect, the invention provides a method for determining the genetic merit of bovine with respect to milk content and volume with reference to a sample of material containing mRNA obtained from the bovine. This method includes ascertaining whether a polymorphism(s) in the sequence of the mRNA encoding DGAT1 is present. The presence of such polymorphisms again indicates an association with altered relative milk lipid and protein production and milk volume.

Again, if an amplification method such as PCR is used in ascertaining whether a polymorphism(s) in the sequence of the mRNA encoding (DGAT1) is present, the method includes reverse transcribing the mRNA using a reverse transcriptase to generate a cDNA and then amplifying the cDNA in the presence of a pair of primers complementary to a nucleotide sequence encoding a protein having biological activity of wild type DGAT1.

In a further aspect, the invention includes the use of a probe in the methods of genotyping according to the invention wherein the probe is selected from any 5 or more contiguous nucleotides of the DGAT1 sequence as shown in Figure 2b, which is therefore sufficiently complementary with a nucleic acid sequence encoding such bovine DGAT1, or its complement, so as to bind thereto under stringent conditions. Diagnostic kits containing such a probe are also included. Such probes may be selected from

ForAA (FAM): CGTTGGCCTTCTTA or DgatADGC (VIC): TTGGCCGCCTTACC. (SEQ ID NOs: 20 and 21 respectively.)

5 The invention further includes isolated nucleic acid molecules encoding the DGAT1 variant proteins i.e. those proteins encoded by SEQ ID NOs: 1 and 4 (Figure 2b), with or without one or more polymorphisms of SEQ ID Nos: 7 to 19 (Table 1), or a fragment or variant thereof. Particularly, the invention includes an isolated nucleic acid molecule comprising a DNA molecule having in whole or in part the nucleotide sequence identified in Figure 2b or which varies from the sequence due to the degeneracy of the
10 genetic code, or a nucleic acid strand capable of hybridising with said nucleic acid molecule under stringent hybridisation conditions.

The invention includes isolated mRNA transcribed from DNA having a sequence which corresponds to a nucleic acid molecule of the invention.

15

The invention includes isolated DNA in a recombinant cloning vector and a prokaryotic or eukaryotic cell containing and expressing heterologous DNA of the invention.

20 The invention includes a transfected cell line which expresses a protein encoded by the nucleic acid molecules of the invention.

The invention also includes a primer composition useful for detection of the presence of DNA encoding DGAT1 and/or the presence of DNA encoding a variant protein. In one form, the composition can include a nucleic acid primer substantially complementary to
25 a nucleic acid sequence encoding DGAT1. The nucleic acid sequence can in whole or in part be that identified in Figure 2b. Diagnostic kits including such a composition are also included.

30 The invention further provides a diagnostic kit useful in detecting DNA encoding a variant DGAT1 protein in bovine which includes first and second primers for amplifying the DNA, the primers being complementary to nucleotide sequences of the DNA upstream and downstream, respectively, of a polymorphism in the portion of the DNA encoding DGAT1 which results in altered relative milk lipid, solid fat content and protein production and milk volume, wherein at least one of the nucleotide sequences is
35 selected to be from a non-coding region of the DGAT1 gene. The kit can also include a third primer complementary to a polymorphism, disclosed herein, located on the DGAT1 gene.

The invention includes a process for producing a protein of the invention, including preparing a DNA fragment including a nucleotide sequence which encodes the protein; incorporating the DNA fragment into an expression vector to obtain a recombinant DNA molecule which includes the DNA fragment and is capable of undergoing replication; transforming a host cell with the recombinant DNA molecule to produce a transformant which can express the protein; culturing the transformant to produce the protein; and recovering the protein from resulting cultured mixture.

Thus in a further aspect, the invention provides a purified protein having biological activity of DGAT1. The terms "isolated" and "purified" as used herein, each refer to a protein substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesised. In certain preferred embodiments, the protein having biological activity of DGAT1 comprises an amino acid sequence and variants shown in Figures 4a and 4b (SEQ ID NOs: 2, 5 and 6). Furthermore, proteins having biological activity of DGAT1 that are encoded by nucleic acids which hybridise under stringent conditions to a nucleic acid comprising a nucleotide sequence shown in Figure 2b (SEQ ID NOs: 1 and 4) are encompassed by the invention.

Proteins of the invention having DGAT1 activity can be obtained by expression of a nucleic acid coding sequence in a suitable host cell using techniques known in the art. Suitable host cells include prokaryotic or eukaryotic organisms or cell lines, for example, yeast, *E. coli*, insect cells and COS1 cells. The recombinant expression vectors of the invention can be used to express a protein having DGAT1 activity in a host cell in order to isolate the protein. The invention provides a method of preparing a purified protein of the invention comprising introducing into a host cell a recombinant nucleic acid encoding the protein, allowing the protein to be expressed in the host cell and isolating and purifying the protein. Preferably, the recombinant nucleic acid is a recombinant expression vector. Proteins can be isolated from a host cell expressing the protein and purified according to standard procedures of the art, including ammonium sulfate precipitation, column chromatography (eg. ion exchange, gel filtration, affinity chromatography, etc.) electrophoresis, and ultimately, crystallisation (see generally "Enzyme Purification and Related Techniques". *Methods in Enzymology*, 22, 233-577 (1971)).

Alternatively, the protein or parts thereof can be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964), or synthesis in homogeneous solution (Houbenwycyl, 1987).

5 It will of course be understood that a variety of substitutions of amino acids is possible while preserving the structure responsible for activity of the DGAT1 proteins disclosed herein. Conservative substitutions are described in the patent literature, as for example, in United States Patent No 5,264,558 or 5,487,983. It is thus expected, for example, that interchange among non-polar aliphatic neutral amino acids, glycine,
10 alanine, proline, valine and isoleucine, would be possible. Likewise, substitutions among the polar aliphatic neutral amino acids, serine, threonine, methionine, asparagine and glutamine could possibly be made. Substitutions among the charged acidic amino acids, aspartic acid and glutamic acid, could probably be made, as could substitutions among the charged basic amino acids, lysine and arginine. Substitutions
15 among the aromatic amino acids, including phenylalanine, histidine, tryptophan and tyrosine would also likely be possible. These sorts of substitutions and interchanges are well known to those skilled in the art. Other substitutions might well be possible. Of course, it would also be expected that the greater percentage of homology ie. sequence similarity, of a variant protein with a naturally occurring protein, the greater
20 the retention of activity.

A further advantage may be obtained through chimeric forms of the proteins, as known in the art. A DNA sequence encoding each entire protein, or a portion of the protein, could be linked, for example, with a sequence coding for the C-terminal portion of *E. coli*
25 β -galactosidase to produce a fusion protein.

The proteins of the invention, or portions thereof, have numerous applications in turn. By way of example, each protein can be used to prepare antibodies which bind to a distinct epitope in an unconserved region of the protein. An unconserved region of the
30 protein is one which does not have substantial sequence homology to other proteins.

Still further, the invention includes an antibody to a bovine DGAT1 variant protein encoded by a nucleotide sequence of the present invention as well as a diagnostic kit containing such an antibody.

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Conventional methods can be used to prepare the antibodies. For example, by using a DGAT1 peptide, polyclonal antisera or monoclonal antibodies can be made using

- standard methods. A mammal, (eg. a mouse, hamster, or rabbit) can be immunised with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunisation can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used to assess the levels of antibodies. Following immunisation, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.
- 10 To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunised animal and fused with myeloma cells by standard somatic cell fusion procedures, thus immortalising these cells and yielding hybridoma cells. Such techniques are well known in the art. For example, the hybridoma technique originally developed by Kohler and Milstein (Kohler, 1975) as well as other
- 15 techniques such as the human B-cell hybridoma technique (Kozbor, 1983) and screening of combinatorial antibody libraries (Huse, 1989). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide, and monoclonal antibodies isolated.
- 20 The term "antibody" as used herein is intended to include fragments thereof which are also specifically reactive with the target protein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to
- 25 reduce disulfide bridges to produce Fab' fragments.
- Another method of generating specific antibodies, or antibody fragments, reactive against the target proteins is to screen expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria, with peptides produced from the
- 30 nucleic acid molecules of the present invention. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries. See for example Ward *et al.*, Huse *et al.*, and McCafferty *et al.* (Ward, 1989); Huse 1989; McCafferty, 1990). Screening such libraries with, for example, a DGAT1 protein can identify immunoglobulin fragments reactive with that DGAT1. Alternatively,
- 35 the SCID-hu mouse developed by Genpharm can be used to produce antibodies, or fragments thereof.

The polyclonal, monoclonal or chimeric monoclonal antibodies can be used to detect the proteins of the invention, portions thereof or closely related isoforms in various biological materials. For example, they can be used in an ELISA, radioimmunoassay or histochemical tests. Thus, the antibodies can be used to quantify the amount and
5 location of a DGAT1 protein of the invention, portions thereof or closely related isoforms in a sample in order to determine the role of DGAT1 proteins. Using methods described hereinbefore, polyclonal, monoclonal antibodies, or chimeric monoclonal antibodies can be raised to non-conserved regions of DGAT1 and used to distinguish a particular DGAT1 from other proteins.

10

The polyclonal or monoclonal antibodies can be coupled to a detectable substance or reporter system. The term "coupled" is used to mean that the detectable substance is physically linked to the antibody. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and
15 radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, and acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and
20 phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S and ^3H . In a preferred embodiment, the reporter system allows quantitation of the amount of protein (antigen) present.

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Such an antibody-linked reported system could be used in a method for determining
25 whether a fluid or tissue sample of a bovine contains a deficient amount or an excessive amount of the relevant DGAT1 protein. Given a normal threshold concentration of such a protein, test kits can be developed.

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The availability of such antibodies gives rise to further applications. One is a diagnostic
30 kit for identifying cells comprising an antibody (such as a monoclonal antibody) which binds to a protein comprising an amino acid sequence shown in Figure 4a and 4b; means for detecting the antibody when bound to the protein, unreacted protein or unbound antibody; means for determining the amount of protein in the sample; and means for comparing the amount of protein in the sample with a standard. In some
35 embodiments of the invention, the detectability of the antibody which binds to a specific DGAT1 protein is activated by the binding (eg. change in fluorescence spectrum, loss of

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radioisotopic label). The diagnostic kit can also contain an instruction manual for use of the kit.

Antibody-based diagnostics are of course not the only possibility. A further diagnostic
5 kit comprises a nucleotide probe complementary to the sequence, or an oligonucleotide
fragment thereof, shown in Figure 2a and 2b, for example, for hybridisation with mRNA
from a sample of cells; means for detecting the nucleotide probe bound to mRNA in the
sample with a standard. In a particular aspect, the kit of this aspect of the invention
10 includes a probe having a nucleic acid molecule sufficiently complementary with a
sequence identified in Figure 2a and 2b, or its complement, so as to bind thereto under
stringent conditions. "Stringent hybridisation conditions" takes on its common
meaning to a person skilled in the art. Appropriate stringency conditions which
promote nucleic acid hybridisation, for example, 6x sodium chloride/sodium citrate
(SSC) at about 45°C are known to those skilled in the art, including in Current
15 Protocols in Molecular Biology, John Wiley & Sons, NY (1989). Appropriate wash
stringency depends on degree of homology and length of probe. If homology is 100%, a
high temperature (65°C to 75°C) may be used. If homology is low, lower wash
temperatures must be used. However, if the probe is very short (<100bp), lower
temperatures must be used even with 100% homology. In general, one starts washing
20 at low temperatures (37°C to 40°C), and raises the temperature by 3-5°C intervals until
background is low enough not to be a major factor in autoradiography. The diagnostic
kit can also contain an instruction manual for use of the kit.

One of the major applications of the present invention is in the marker assisted
25 selection of bovines having a polymorphism in the DGAT1 gene and which are
associated with improved milk production traits. The invention therefore provides a
diagnostic kit which can be used to determine the DGAT1 genotype of bovine genetic
material, for example. One kit includes a set of primers used for amplifying the genetic
material. A kit can contain a primer including a nucleotide sequence for amplifying a
30 region of the genetic material containing one of the polymorphisms described herein.
Such a kit could also include a primer for amplifying the corresponding region of the
normal DGAT1 gene, i.e. the sequence without polymorphisms. Usually, such a kit
would also include another primer upstream or downstream of the region of interest
complementary to a coding and/or non-coding portion of the gene. These primers are
35 used to amplify the segment containing the mutation, i.e. polymorphism, of interest.

In particular, the invention is directed to the use of the polymorphisms in the DGAT1 gene in the genotyping of cows and bulls as well as to cows and bulls selected by such genotyping which have one or more of said polymorphisms in the DGAT1 gene. Such bulls so selected are of valuable breeding stock and the invention is also directed to the semen produced by such selected bulls for breeding purposes. Cows so selected are also useful as breeding stock as are their offspring. In addition, such cows may produce valuable dairy herds as the milk produced by such cows is produced in greater volumes than equivalent non-selected cows, and/or has an altered composition in that it comprises less milkfat and more milk protein. Such milk and products made therefrom also form part of the invention. It is also noted that the milk from these selected cows will be valuable as the fat content is not only decreased but is also characterised by being softer. Without being bound by theory, it is thought that this increased fat softness is due to the fatty acid composition being such that there is less saturated and more unsaturated fat in the milk of selected cows. Thus it is anticipated that products made from such milk will have processing advantages, such as in the production of more spreadable butter, as well as having a health benefit on consumers, as generally unsaturated fats are considered to be more "healthy" than saturated fats. The protein composition of milk produced by such selected cows is also altered. In particular, such milk comprises an altered protein yield compared to milk for non-selected cows and the casein:whey ratio is also altered which makes such milk valuable for cheese production.

Thus, the present invention involves genotyping bovine, both cows and bulls, for the DGAT1 polymorphisms disclosed herein, selected cows and bulls so genotyped, milk and semen produced by the selected cows and bulls so genotyped, offspring produced by the selected bovine, including embryos and cells (including cell lines) useful for cloning said selected bovine.

The actual genotyping is carried out using primers that target specific polymorphisms as described herein and that could function as allele-specific oligonucleotides in conventional hybridisation, Taqman assays, OLA assays, etc. Alternatively, primers can be designed to permit genotyping by microsequencing.

One kit of primers can include first, second and third primers, (a), (b) and (c), respectively. Primer (a) is based on a region containing a DGAT1 mutation such as described above. Primer (b) encodes a region upstream or downstream of the region to be amplified by primer (a) so that genetic material containing the mutation is amplified,

by PCR, for example, in the presence of the two primers. Primer (c) is based on the region corresponding to that on which primer (a) is based, but lacking the mutation. Thus, genetic material containing the non-mutated region will be amplified in the presence of primers (b) and (c). Genetic material homozygous for the DGAT1 gene will thus provide amplified products in the presence of primers (b) and (c). Genetic material homozygous for the mutated gene will thus provide amplified products in the presence of primers (a) and (b). Heterozygous genetic material will provide amplified products in both cases.

- 10 The present invention also contemplates the modulation of milk production and content in non-human animals by modulating the activity of the DGAT1 protein. In particular, this aspect of the invention includes a method of modulating milk production and/or milk content in a lactating bovine, the method comprising administering to the bovine an effective amount of a nucleic acid molecule substantially complementary to at least a portion of mRNA encoding the bovine DGAT1 variant proteins and being of sufficient length to sufficiently reduce expression of said DGAT1, i.e. by use of antisense nucleic acids.

- Antisense nucleic acids or oligonucleotides (RNA or preferably DNA) can be used to inhibit DGAT1 production in a bovine if this is considered desirable e.g. in order to produce a bovine capable of improved milk production, i.e. increased milk volume and decreased milkfat content. Antisense oligonucleotides, typically 15 to 20 bases long, bind to the sense mRNA or pre mRNA region coding for the protein of interest, which can inhibit translation of the bound mRNA to protein. The cDNA sequence encoding DGAT1 can thus be used to design a series of oligonucleotides which together span a large portion, or even the entire cDNA sequence. These oligonucleotides can be tested to determine which provides the greatest inhibitory effect on the expression of the protein (Stewart 1996). The most suitable mRNA target sites include 5'- and 3'-untranslated regions as well as the initiation codon. Other regions might be found to be more or less effective.

Alternatively, an antisense nucleic acid or oligonucleotide may bind to DGAT1 coding sequences.

- 35 In yet another embodiment, the invention provides a method of modulating milk production and/or milk content in a lactating bovine, including administering to the bovine an effective amount of a nucleic acid molecule having ribozyme activity and a

nucleotide sequence substantially complementary to at least a portion of mRNA encoding a bovine DGAT1 and being of sufficient length to bind selectively thereto to sufficiently reduce expression of said DGAT1.

- 5 Rather than reducing DGAT1 activity in the bovine by inhibiting gene expression at the nucleic acid level, activity of the relevant DGAT1 protein may be directly inhibited by binding to an agent, such as, for example, a suitable small molecule or a monoclonal antibody.
- 10 Thus, the invention also includes a method of inhibiting the activity of bovine DGAT1 in a lactating bovine so as to modulate milk production and/or milk solids content, comprising administering an effective amount of an antibody to the relevant DGAT1.

The invention still further includes a method of modulating milk production and/or
15 milk solids content by raising an autoantibody to a bovine DGAT1 in the bovine. Raising the autoantibody can include administering a protein having DGAT1 activity to the bovine.

In still a further embodiment, nucleic acids which encode DGAT1 proteins can be used
20 to generate transgenic animals. A transgenic animal (eg. a mouse) is an animal having cells that contain a transgene, which transgene is introduced into the animal or an ancestor of the animal at a prenatal, eg. an embryonic stage. A transgene is DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, a bovine cDNA, comprising the nucleotide sequence shown in Figure 2b,
25 or an appropriate variant or subsequence thereof, can be used to generate transgenic animals that contain cells which express the relevant DGAT1. Likewise, variants can be used to generate transgenic animals. "Knock out" animals can also be generated.

Methods for generating transgenic animals, particularly animals such as mice, have
30 become conventional in the art are described, for example, in US Patent Nos. 4,736,866 and 4,870,009. In such methods, plasmids containing recombinant molecules are microinjected into mouse embryos. In particular, the plasmids can be microinjected into the male pronuclei of fertilised one-cell mouse eggs; the injected eggs transferred to pseudo-pregnant foster females; and the eggs in the foster females allowed to develop to
35 term. (Hogan, 1986). Alternatively, an embryonal stem cell can be transfected with an expression vector comprising nucleic acid encoding a DGAT1 protein, and cells containing the nucleic acid can be used to form aggregation chimeras with embryos

from a suitable recipient mouse strain. The chimeric embryos can then be implanted into a suitable pseudopregnant female mouse of the appropriate strain and the embryo brought to term. Progeny harbouring the transfected DNA in their germ cells can be used to breed uniformly transgenic mice.

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Such animals could be used to determine whether a sequence related to an intact DGAT1 gene retains biological activity of the encoded DGAT1. Thus, for example, mice in which the murine DGAT1 gene has been knocked out and containing the nucleic acid sequence identified in Figure 2b or fragment or variant thereof could be generated. The animals could be examined with reference to milk production and content.

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The pattern and extent of expression of a recombinant molecule of the invention in a transgenic mouse is facilitated by fusing a reporter gene to the recombinant molecule such that both genes are co-transcribed to form a polycistronic mRNA. The reporter gene can be introduced into the recombinant molecule using conventional methods such as those described in Sambrook *et al.*, (Sambrook, 1989). Efficient expression of both cistrons of the polycistronic mRNA encoding the protein of the invention and the reporter protein can be achieved by inclusion of a known internal translational initiation sequence such as that present in poliovirus mRNA. The reported gene should be under the control of the regulatory sequence of the recombinant molecule of the invention and the pattern and extent of expression of the gene encoding a protein of the invention can accordingly be determined by assaying for the phenotype of the reporter gene. Preferably the reporter gene codes for a phenotype not displayed by the host cell and the phenotype can be assayed quantitatively. Examples of suitable reporter genes include *lacZ* (β -galactosidase), *neo* (neomycin phosphotransferase), *CAT* (chloramphenicol acetyltransferase) *dhfr* (dihydrofolate reductase), *aphIV* (hygromycin phosphotransferase), *lux* (luciferase), *uidA* (β -glucuronidase). Preferably, the reporter gene is *lacZ* which codes for β -galactosidase. β -galactosidase can be assayed using the lactose analogue X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) which is broken down by β -galactosidase to a product that is blue in colour.

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Still further transgenic applications of the invention arise from knocking out the endogenous gene encoding DGAT1 in mammals and replacing this with a transgene, in order to obtain a desired effect. This is particularly true in cattle raised for milk production. For example, additional copies of the gene encoding DGAT1 can be inserted as a transgene, or the endogenous gene associated with a high level expression promoter in a transgene. It may also prove advantageous to substitute a defective gene

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rather than delete the entire sequence of DNA encoding for a protein having DGAT1 activity. A method of producing a transgenic bovine or transgenic bovine embryo is described in United States Patent No. 5,633,076, issued 27 May 1997, for example.

- 5 These transgenic animals of the invention can again be used to investigate the molecular basis of DGAT1 action. For example, it is expected that mutants in which one or more of the conserved cysteine residues has been deleted would have diminished activity in relation to a DGAT1 protein in which all such residues are retained. Further, deletion of a proteolytic cleavage site would likely result in a mutant lacking biological
10 activity of DGAT1.

Transgenic animals of the invention can also be used to test substances for the ability to prevent, slow or enhance DGAT1 activity. A transgenic animal can be treated with the substance in parallel with an untreated control transgenic animal. Substances
15 which could be tested in this way include proteins extracted from foods ingested by the animal. For example, proteins extracted from pastoral grasses and other fodder can be tested to determine their effect on DGAT1 activity, including to determine whether breed-specific effects can be induced.

- 20 Thus, in further aspects, the invention provides transgenic non-human animals. These include by way of example only a transgenic bovine having a genome lacking a gene encoding a protein having biological activity of DGAT1 (or indeed any DGAT1 activity at all); a transgenic mouse having a genome containing a gene encoding a bovine protein having biological activity of any DGAT1; and a transgenic bovine having a gene
25 encoding a bovine protein having biological activity of a bovine DGAT1 and heterologous nucleotide sequence antisense to the gene. The transgenic bovine can include a gene encoding a nucleic acid sequence having ribozyme activity and in transcriptional association with the nucleotide sequence antisense to the gene.

- 30 The invention further provides a transgenic bovine having a genome which includes additional copies of a gene encoding a protein having biological activity of DGAT1 or copies of a gene encoding a protein having biological activity of DGAT1 under control of a high expression promoter.

- 35 These are but a selection of the applications of this invention. Others will be apparent to those persons skilled in this art and are in no way excluded. To the contrary, the

invention extends to cover not only the specific teaching provided but also all variations and modifications which are within the skill and contemplation of the addressee.

The invention will now be defined by specific examples which are illustrative only and are not intended to limit the invention in any way.

EXPERIMENTAL

1. Location of the gene responsible for the observed QTL

Construction of a BAC contig spanning the BULGE9-BULGE30 interval.

In order to clone the gene(s) responsible for the observed QTL effect, a BAC contig spanning the corresponding marker interval was constructed. This was accomplished by screening a BAC library by filter hybridisation with the microsatellite markers available for proximal BTA14q, as well as with human cDNA clones mapping to the orthologous chromosome segment on the human RH transcript map: 8q23.3-ter (Riquet *et al.*, (1999)). The ends of the isolated BACs were sequenced, sequence tagged sites (STS) developed from the corresponding sequences, and mapped onto a bovine x hamster whole genome radiation hybrid panel. This STS content mapping approach lead to the construction of the BAC contig shown in Figure 1.

DGAT1 maps to the BULGE9-BULGE30 interval and is a strong positional candidate for the QTL.

A murine gene encoding a protein with Diacylglycerol-o-acyltransferase (DGAT1) activity was identified (Cases *et al.*, (1998)) and shown to completely inhibit lactation when knocked out in the mouse (Smith *et al.*, (2000)). This gene was reported in the human to map to HSA8qter (Cases *et al.*, (1998)), ie. in the region orthologous to that containing the bovine QTL. Screening the publicly available databases with the published murine and human DGAT1 cDNA sequences allowed identification of (i) a human BAC clone containing the human DGAT1 gene (AF205589), and (ii) three bovine Expressed Sequence Tags (AW446908; AW446985; AW652329) jointly covering approximately two thirds of the bovine gene. Aligning the human DGAT1 genomic sequences with the human and bovine cDNA sequences allowed the corresponding intron-exon boundaries to be identified. Primers were developed to PCR amplify a portion of the bovine DGAT1 gene. Screening the BACs composing the BULGE9-BULGE30 contig clearly indicated that the bovine DGAT1 gene was contained in a

subset of the BACs allowing us to accurately position the DGAT1 gene in the contig of Figure 1.

These results demonstrated that the map position of DGAT1 coincided with the most likely position of the chromosome 14 QTL as determined by linkage and linkage disequilibrium analyses. Knowing that the QTL primarily affects fat content, knowing the enzymatic activity of DGAT1 and the effect of a DGAT1 knock-out on lactation, this gene was considered to be a very strong positional candidate for the corresponding QTL.

10 *Organisation of the bovine DGAT1 gene*

The organisation of the bovine DGAT1 gene was determined by sequence analysis of one of the DGAT1 containing BACs. Primers were designed based on the available bovine, murine and human cDNA sequences which were either used for direct sequencing of the BAC clone or to generate PCR products corresponding to different parts of the bovine DGAT1 gene from this BAC which were then subjected to cycle-sequencing. All available sequences were then merged using the Phred / Phrap software (Ewing *et al.*, (1998); Ewing & Green, (1998); Gordon *et al.*, (1998)) to yield the consensus sequence shown in Figures 2a and b.

20 RT-PCR, 5' and 3' RACE experiments were performed on mRNA isolated from bovine mammary gland and the obtained PCR products subjected to cycle sequencing. Comparison of the genomic and cDNA sequences showed that the bovine *DGAT1* gene spans 8.6 Kb and comprises 17 exons measuring 121.8 bp on average (range: 42 – 436 bp) and allowed intron-exon boundaries to be identified (Figures 2a, 2b and 3). The cDNA sequence is also set out in SEQ ID NO: 4. While the first two introns are respectively 3.6 and 1.9 Kb long, the remaining 14 introns are only 92.4 bp long on average (range: 70 – 215 bp). All introns conform to the GT-AG rule and are strictly conserved between human and bovine. The bovine *DGAT1* gene is transcribed in a mRNA comprising >31 bp of 5' UTR sequence (Figure 2a), 1470 bp coding for a protein of 489 amino-acids, and 275 bp of 3' UTR sequence including a canonical AATAAA polyadenylation signal. The human and bovine *DGAT1* nucleotide (coding) and protein sequences are respectively 89.5% and 92.5% identical (Figures 2a, 2b, 4a and 4b). In addition, an alternative splicing variant is predicted in the bovine for exon VIII (Figure 2b). The corresponding bovine cDNAs are predicted to encode proteins comprising 35 respectively 489 and 467 (alternative splicing variant) amino-acid residues (Figures 4a and 4b).

The predicted "Q" and "q" QTL alleles differ by a non conservative lysine to alanine amino-acid substitution in the DGAT1 gene.

Assuming that DGAT1 is indeed the QTL, it is predicted that the identified "Q" and "q" QTL alleles will correspond to functionally distinct DGAT1 alleles, ie. will differ at one or more mutations causing these alleles to be functionally different. To test this hypothesis, the structure of the DGAT1 gene in individuals predicted to be of different QTL genotypes: "QQ", "Qq" and "qq" was examined. More specifically, the DGAT1 gene from:

- 10 (i) two sires with " H^{Q-D}/h^q " genotype as well as two of their " H^{Q-D}/H^{Q-D} " offspring, two of their " h^q/h^q " offspring and one " H^{Q-D}/h^q " offspring, and
- (ii) one " H^{Q-NZ}/h^q " sire with one of its " H^{Q-NZ}/H^{Q-NZ} " offspring

15 was analysed wherein H^{Q-D} corresponds to the Dutch Q haplotype and H^{Q-NZ} corresponds to the New Zealand Q haplotype, and primer pairs were designed that allowed for the amplification from genomic DNA of (i) the coding portion of exon I, (ii) exon II, and (iii) the chromosome regions spanning exons III to XVII. The corresponding PCR products from the selected individuals were cycle-sequenced and the resulting sequences examined with the *Polyphred* software.

Additional sequencing analysis, as described above, on DNA from a range of breeds revealed additional polymorphisms included in Table 1 (see Methods section for breeds). Four such polymorphisms were investigated further:

25 (i) **K232A**: a substitution of a ApA by a GpC dinucleotide in exon VIII (respectively positions 694 and 695 counting from the start codon in the cDNA). The substitution of these two adjacent nucleotides results in a non conservative lysine (hydrophylic basic amino acid) to alanine (hydrophobic amino acid) substitution in the DGAT1 protein. The lysine residue affected by this polymorphism is conserved in the human and murine DGAT1 sequences. Together with the resulting change in the electrical charge of the protein, this strongly suggests that this amino-acid substitution is likely to result in a functional difference between the two corresponding alleles and to be at least partly responsible for the observed QTL effect.

35 (ii) **Nt984+8(Base 7438 A-G)**: A A to G substitution in intron 12, eight base pairs downstream of exon XII. Following standard nomenclature, this polymorphism will be

referred to as *Nt984+8(A-G)*. This polymorphism cannot be predicted as such to modify the functionality of the corresponding alleles although an effect on the splicing mechanism cannot be excluded given its proximity to the intron-exon boundary.

- 5 *(iii) Nt984+26(Base 7456 C-T)*: A C to T substitution in intron 12, 26 base pairs downstream of exon XII. Following standard nomenclature, this polymorphism will be referred to as *Nt984+26(Base 7456 C-T)*. Again, this polymorphism cannot be predicted as such to modify the functionality of the corresponding alleles although an effect on the splicing mechanism cannot be excluded given its proximity to the intron-exon
10 boundary.

- 15 *(iv) Nt1470+85(Base 8402 C-T)*: A C to T substitution in the 3' UTR. Following standard nomenclature, this polymorphism will be referred to as *Nt1470+85(Base 8402 C-T)*. Again, this polymorphism cannot be predicted as such to modify the functionality of the corresponding alleles although an effect on polyadenylation or mRNA stability cannot be excluded.

Conclusion

- 20 These four polymorphisms were shown to assort into three distinct SNP haplotypes referred to as *sHQ-D*, *sHQ-NZ* and *shg* because in the sequenced samples they coincided respectively with microsatellite haplotypes $\mu HQ-D$, $\mu HQ-NZ$ and μhg . The base pair compositions of these three SNP haplotypes are shown in Figure 3.
- 25 Because the *sHQ-NZ* and *shg* marker haplotypes share the G residue at the *DGAT1* *Nt984+8(Base 7438 A-G)* site, the causality of this polymorphism in the determinism of the QTL could be excluded. For the three remaining polymorphic sites, however, the *DGAT1* haplotypes associated with marker haplotypes *sHQ-D* and *sHQ-NZ* proved identical to each other while different from the *shg* *DGAT1* haplotype. Either of these three
30 polymorphisms could therefore be responsible for the observed QTL effect. The *Nt984+26(Base 7456 C-T)* and *Nt1470+85(Base 8402 C-T)* polymorphisms are *a priori* more likely to be neutral with respect to *DGAT1* activity because of their respective location in an intron and the 3' UTR and likewise the other non coding or neutral polymorphism shown in Table 1. A direct effect of the *K232A* mutation on *DGAT1*
35 activity, however, is very plausible. Indeed, the corresponding lysine residue is conserved amongst all examined mammals (i.e. human, mouse, rat, pig, sheep, bison) demonstrating its functional importance (Figure 5).. The evolutionary conservation of

this lysine residue also demonstrates that the *K* residue characterizing the *sHQ^D* and *sHQ^{NZ}* marker haplotypes is more than likely the ancestral state and that it is the *A* residue characterizing the *sh^a* haplotypes that corresponds to a more recently evolved state.

5.

2. Genotype Testing and Analysis I

This summarises the genotype testing and subsequent analysis of Holstein-Friesian animals sourced from New Zealand and Holland which were tested for the presence of the K232A polymorphism. Reference to allele "Q" corresponds to the *K* residue and allele "q" to the *A* residue (as shown in Figure 3 and Table 1).

An oligonucleotide ligation assay (OLA) was developed as described in the method section below that allows for efficient genotyping of the four *DGAT1* polymorphisms simultaneously. This OLA-test was used to genotype a previously described (Farnir et. al., 2000) "grand-daughter design" (i.e. series of paternal half-brother pedigrees) comprising 1,818 Dutch Holstein-Friesian sires as well as a "daughter design" (i.e. series of paternal half-sister pedigrees) comprising 529 New Zealand Holstein-Friesian cows selected according to phenotype as described below. The marker linkage phase for each individual was determined as described below.

Fig. 6 summarizes the frequency distribution of *DGAT1* haplotypes encountered in the Dutch and New Zealand populations respectively. Four distinct *SNP* haplotypes were identified. Three of these correspond to the *sHQ^D*, *sHQ^{NZ}* and *sh^a* that were previously identified by sequencing, and jointly account for 99% and 98% of the chromosomes in the Dutch and New-Zealand populations respectively. A fourth minor haplotype was found accounting for the remaining 1% and 2% of the chromosomes. As this haplotype codes for a *K* residue at position 232 it was assumed to correspond to a fat increasing "Q" allele and was therefore referred to as *sHQ^{III}* (Fig. 3). The observation that the *K* residue is found on three distinct *DGAT1* haplotypes while the *A* residue is found on a unique *DGAT1* haplotype is in agreement with *K* being the more ancient state.

The *sHQ^D* and *sHQ^{NZ}* *SNP* haplotypes (coding for a *K* residue at position 232) appear to be in strong linkage disequilibrium (LD) with the flanking microsatellite markers *BULGE09* and *BULGE11*, as they are in essence associated with unique microsatellite haplotypes corresponding respectively to the previously defined *μHQ^D* and *μHQ^{NZ}* haplotypes (Fig. 6C&D). In sharp contrast, the *sh^a* haplotype (coding for an *A* residue at

position 232) is nearly evenly distributed across more than ten distinct microsatellite haplotypes (Fig. 6B).

These observations are in excellent agreement with the results of the combined linkage and LD analysis (Fernier et. al., 2000). These studies indeed predicted (i) that in the Dutch population the vast majority (estimates ranging from 81% to 92%) of "Q" allele (= K) would reside on the μH^{Q-D} microsatellite haplotype, (ii) that in the New Zealand population a large fraction (estimates ranging from 36% to 51%) of "Q" alleles would reside on haplotype μH^{Q-NZ} (we now see that the remainder correspond mainly to the μH^{Q-D} microsatellite haplotype) and (iii) that in both populations the "q" alleles (= A) would correspond to multiple marker haplotypes, corresponding to h^a .

Figure 7 illustrates the gain in LD signal that could be obtained in the Dutch Holstein-Friesian grand-daughter design when adding the *DGAT1* polymorphisms to the previously available markers for proximal BTA14q and performing a joint linkage and LD multipoint analysis (Fernier et. al., 2000) using the sires "daughter yield deviations" (DYD (Van Raden and Wiggans, 1991) corresponding to half breeding values) for milk fat percentage as phenotype. It can be seen that the lod score attributable to LD essentially doubles (from 3.7 to 7.8), and maximizes exactly at the position of the *DGAT1* gene. This result strongly supports the causal involvement of the *DGAT1* gene in the QTL effect. The corresponding ML estimates of the "Q" to "q" allele substitution effect ($\alpha/2$) (as defined in Falconer and Mackay, 1996), residual standard deviation (σ), population frequency of the "Q" allele (f_Q), number of generations to coalescence (g) and heterogeneity parameter (ρ) were respectively 0.11% ($\alpha/2$), 0.06% (σ), 0.20 (f_Q), 5 (g) and 0.84 (ρ).

Using the same Dutch Holstein-Friesian population, the additive effect of the *DGAT1* K232A polymorphism on milk yield and composition was examined. The sons DYDs for milk yield (kgs), protein yield (kgs), fat yield (kgs), protein percentage and fat percentage, were analysed using a mixed model including (i) a regression on the number of K alleles in the genotype (0, 1 or 2), and (ii) a random polygenic component estimated using an individual animal model and accounting for all known pedigree relationships. Table 2 below, reports the obtained results. It can be seen that the K232A mutation has an extremely significant effect on the five analysed dairy traits. The proportion of the trait variance explained by this polymorphism in this population ranges from 8% (protein yield) to 51% (fat percentage), corresponding to between 10% (protein yield) and 64% (fat percentage) of the genetic variance (= QTL + polygenic).

Note that the proportion of the variance explained by the full model ($1-r^2_{\text{error}}$) is of the order of 70% for the yield traits and 80% for the percentage traits, which is in agreement with the known reliabilities of the corresponding DYDs (Van Raden and Wiggans, 1991). An interesting feature of this QTL effect is that the “q” to “Q” substitution increases fat yield, while decreasing milk and protein yield, despite the strong overall positive correlation characterizing the three yield traits.

Table 2:

Effect of the *DGAT1* K232A mutation on sire's daughter yield deviations (DYDs) for milk yield and composition.

Trait	$\alpha/2$	r^2_{QTL}	p-value QTL	$r^2_{\text{polygenic}}$	r^2_{error}
Milk yield (Kgs)	-158 Kgs	0.18	5.00E-35	0.49	0.32
Fat yield (Kgs)	5.23 Kgs	0.15	1.57E-29	0.55	0.30
Protein yield (Kgs)	-2.82 Kgs	0.08	1.70E-15	0.65	0.26
Fat %	0.17 %	0.51	4.33E-122	0.29	0.19
Protein %	0.04 %	0.14	5.05E-28	0.66	0.20

(i) $\alpha/2$: QTL allele substitution effect on DYD (half breeding value), corresponding in the mixed model to the regression coefficient on the number of K alleles in the *DGAT1* K232A genotype, and to $\alpha/2$, where α is defined according to ref. Falconer and Mackay, 1996. (ii) r^2_{QTL} : proportion of the trait variance explained by the *DGAT1* K232A polymorphism. (iii) p-value QTL : statistical significance of the *DGAT1* K232A effect. (iv) $r^2_{\text{polygenic}}$: proportion of the trait variance explained by the random, polygenic effect in the mixed model. (v) r^2_{error} : proportion of the trait variance unexplained by the model.

The two previous analyses examined the effect of the *DGAT1* polymorphism on estimated breeding values. By definition, this phenotype will only account for the additive component of the *DGAT1* effect, and justifies the use of a regression on the number of K alleles in the mixed model. To evaluate the dominance relationship between the *DGAT1* alleles, the effect of the K232A genotype on the lactation values (first yield deviations (Van Raden and Wiggans, 1991)) of the cows composing the New Zealand daughter design were analysed. This was achieved by using a mixed model including (i) a fixed effect corresponding to the K232A genotype, and (ii) a random polygenic component accounting for all known pedigree relationships (“animal model”). Very significant effects of K232A genotype on all examined yield and composition traits were found in this population as well (Table 3, below), accounting for between 1% (protein yield) and 31% (fat percentage) of the trait variance. The observed dominance deviations, d , corresponding to the difference between the genotypic value of the KA

genotype and the midpoint between the AA and KK genotypic values (Falconer and Mackey, 1996) are shown in Table 3 below. Genotypic values of the heterozygous genotype are systematically in between alternate homozygotes. None of the d -values proved to be significantly different from zero, indicating an absence of dominance.

5 Average K to A QTL allele substitution effects, α (Falconer and Mackey, 1996), were computed from the estimates of a - and d -values, as well as the population frequencies of the K and A alleles (Table 3). The predicted substitution effects are in good agreement with those computed from the grand-daughter design: the K allele increases fat yield, fat % and protein %, while decreasing milk and protein yield. The absolute

10 values of α estimated from the grand-daughter and daughter design are in perfect agreement for fat and protein %, while for the yield traits estimates are larger in the grand-daughter design when compared to the daughter design. The exact reasons for this are being explored. It could be due to the fact that the sire population in the grand-daughter design is not representative of the cow population in general, or to

15 intrinsic differences between the Dutch and New-Zealand populations and/or environment.

Table 3:

Effect of the DGAT1 K232A mutation on cows' lactation values

for milk yield and composition.

Trait	a	d	α	r^2_{QTL}	p-val QTL	$r^2_{polygenic}$	r^2_{error}
Milk yield (Kgs)	-144 Kgs	-42 Kgs	-161 Kgs	0.03	1.05E-8	0.54	0.43
Fat yield (Kgs)	7.82 Kgs	-0.89 Kgs	7.46 Kgs	0.09	1.77E-20	0.46	0.45
Protein yield (Kgs)	-2.34 Kgs	-0.76 Kgs	-2.64 Kgs	0.01	4.35E-2	0.37	0.42
Fat %	0.41 %	0.03 %	0.42%	0.31	2.5E-108	0.49	0.20
Protein %	0.08%	0.03 %	0.08%	0.04	1.60E-20	0.72	0.24

(i) a : half the difference between the genotypic values of the KK and AA genotypes (Falconer and Mackey, 1996). (ii) d : dominance deviation (Falconer and Mackey, 1996): deviation of the KA genotypic value from the midpoint between the AA and KK genotypic values; none of these proved to be significantly different from zero. (iii) α : average K to A substitution effect, computed as " $a + d(q-p)$ " (Falconer and Mackey, 1996), where q is the allelic frequency of K ($=0.7$) and p of A ($=0.3$) (iv) r^2_{QTL} : proportion of the trait variance explained by the DGAT1 K232A polymorphism. (v) p-val QTL : statistical significance of the DGAT1 K232A effect. (vi) $r^2_{polygenic}$: proportion of the trait variance explained by the random, polygenic effect in the mixed model. (vii) r^2_{error} : proportion of the trait variance unexplained by the model.

Pedigree material and phenotypes. The pedigree material used for the association studies comprised a "grand-daughter design" (Weller et. al., 1990) counting 1,818 Holstein-Friesian bulls sampled in the Netherlands, as well as a "daughter-design" (Weller et. al., 1990) counting 529 Holstein-Friesian cows sampled in New Zealand. The phenotypes of the sires were "daughter yield deviations" (DYD: unregressed weighted averages of the daughter's lactation performances adjusted for systematic environmental effects and breeding values of the daughter's dams and expressed as deviations from the population mean (Van Raden and Wiggans, 1991)) obtained directly from CR-Delta (Arnhem - The Netherlands). The phenotypes of the cows were "lactation values" (first lactation yield deviations (YD), i.e. weighted average lactation performances expressed as deviations from the population mean, adjusted for management group, permanent environmental effects and herd-sire interaction effects (Van Raden and Wiggans, 1991)) obtained directly from Livestock Improvement Corporation (Hamilton - New Zealand).

Combined linkage and linkage disequilibrium analysis and association studies. The maximum likelihood procedure for combined linkage and linkage disequilibrium analysis is described in detail in Farnir, 2000. The association study in the grand-daughter design was performed using the following model:

$$y_i = \mu + \beta x_i + a_i + e_i$$

where y_i is the DYD of son i , μ is the overall population mean, β is a fixed regression coefficient estimating the A to K allele substitution effect, x_i is an indicator variable corresponding to the number of K alleles in the K232A genotype, a_i is a random polygenic component accounting for all known pedigree relationships ("animal model" Lynch and Walsh, 1997) and e_i is a random residual. The association study in the daughter design was performed using the model:

$$y_i = \mu + g_i + a_i + e_i$$

where y_i is the lactation value of cow i , g_i is a fixed effect corresponding to the DGAT1 genotype (KK, KA, or AA), a_i is a random polygenic component accounting for all known pedigree relationships ("animal model" Lynch and Walsh, 1997) and e_i is a random residual. In both instances, maximum likelihood solutions for β , g_i , a_i , e_i , σ^2_a , σ^2_e were obtained using the MTDFREML program (Boldman et al, 1997).

3. Genotype Testing and Analysis II

5 This summarises the genotype testing and subsequent analysis of Holstein-Friesian, Jersey and Ayrshire animals in a separate population from those presented in genotype testing and analysis I, above.

Progeny tested sires

10 Each year Livestock Improvement Corporation (New Zealand) progeny test some 200-300 bulls per year. This entails the bulls being genetically evaluated on the basis of 50-85 daughters per sire. The sires are evaluated for milk fat, milk protein, milk volume and 20 non-production traits. Semen has been retained from all progeny tested sires since the early 1970s. DNA was extracted from the semen and genotyped for the K232A DGAT1 polymorphism using the 7900 Taqman system (see Methods section below).

15 Statistical analysis was undertaken on this dataset using Restricted Maximum Likelihood (REML) and the average information algorithm (Johnson and Thompson, 1995). The linear model included the fixed effects of DGAT1 (3 classes; 0, 1 and 2 copies of the Q allele i.e. the K residue) and a covariate corresponding to the proportion of overseas genetics. The random effect was animal with a relationship matrix based on all known relationships. Daughter yield deviations (DYDs), weighted averages of a sire's daughter's lactation performances expressed as deviations from the population mean (van Raden and Wiggans 1991) were used as the phenotypic measurement. The phenotypes were weighted by a weighting factor based on the variance of the DYD for a son being:

$$\text{Var DYD} = \left[\frac{1 + (n-1) \frac{1}{4} h^2}{n} \right] \sigma_p^2$$

30 where Var DYD is the variance of son's DYD; n is the number of daughters contributing to the DYD; h^2 is the heritability, which was taken as 0.35 for yield traits.

The dataset was analysed separately for the 3 major breeds; Holstein-Friesian, Jersey and Ayrshire.

Seventeen hundred and thirteen Holstein-Friesian sires were included in the analysis. The effect of the DGAT1 polymorphism was extremely significant for the three milk production traits (Table 4). With each additional Q allele the level of milk fat production increases by approximately 6 kg per lactation, milk protein production decreases by approximately 2.5 kg per lactation and milk volume decreases by approximately 125 litres per lactation.

Table 4:
Effect of the DGAT1 polymorphism on milk production in the Holstein-Friesian bull population (kilograms per lactation).

	Fat	Protein	Milk
qq	0	0	0
Qq	6.86	-2.13	-128
QQ	11.83	-4.80	-266
st. error	0.87	0.68	24

The effects for the Jersey and Ayrshire breeds were less significant than those of the Holstein-Friesian breed but were consistent in direction of effects.

Daughters for milk components

Data collection was integrated with LIC's herd testing service using a sample of 102 herds involved in LIC's Sire Proving Scheme (SPS) in 1995. In addition to milk volume from herd testing, the concentrations of fat, crude protein (total nitrogen), casein, whey and lactose were determined. The data was collected from over 3,000 cows born in 1996 and first calving in the 1998 spring season, these being predominantly the daughters of approximately 220 SPS bulls. The milk characteristics were measured at three herd tests on each cow, with each herd having a herd test in each of the Sept/Oct, Nov/Dec and Jan/Feb periods. The Milkoscan FT120, which employs Fourier transform infrared spectrophotometry with enhanced milk calibrations (Foss Electric Application Note Nos. 95, P/N 492280 and 102, P/N 578377), was used to determine the milk component concentrations.

Nine hundred and twelve daughters were genotyped for the DGAT1 polymorphism using the OLA system. Analysis was undertaken using SAS (Statistics, Version 5, 1985) fitting a general linear model. The model included sire and maternal grandsire as fixed effects, DGAT1 polymorphism (3 classes; 0, 1 and 2 copies of the Q allele), covariates including 16ths of Holstein-Friesian, Jersey, Ayrshire and other, proportion of overseas

genetics within the Holstein-Friesian, Jersey and Ayrshire breeds. Yield deviations that were pre-adjusted for herd, stage of lactation among other fixed effects were used (Johnson et al 2000).

- 5 The DGAT1 polymorphism is statistically significant for Lactose, casein, beta-casein and whey yield and also for casein and beta-casein percent as outlined in Table 5.

Table 5:
Effect of the DGAT1 polymorphism on milk components.

10

Trait	qq	Qq	QQ	p-value
Lactose yield*	48	23	0	<0.0001
Casein yield*	11.0	5.8	0	0.01
Casein %	-0.13	-0.06	0	<0.0001
Whey yield*	6.86	2.31	0	<0.0001
β -casein yield*	3.98	2.19	0	0.05
β -casein %	-0.43	-0.23	0	0.0001

* Units = g/day for lactose, casein and whey yield and g/litre for β -casein yield

Daughters for solid fat content

- 15 Six hundred and ninety-two daughters were phenotyped for solid fat content. Solid fat content of the milkfat is a characteristic which has a major influence on the functionality of milkfat products, and in particular has a significant effect on the hardness of butter (MacGibbon & McLennan, 1987). The solid fat content at 10°C (SFC 10) was used for comparison of the properties of the milkfat as it relates well to the
- 20 sectility hardness measurement of butter, a major functional property. Thus the performance of milkfat products may be predicted from the characteristics of the milk produced. The solid fat content (SFC) of the extracted fat was determined by pulsed nuclear magnetic resonance (NMR) and expressed as percentage solid fat (MacGibbon & McLennan, 1987). As the milkfat was melted to remove any thermal history, prior to
- 25 recrystallization under standard conditions, the SFC simply reflects the chemical composition of the milkfat.

The 692 daughters were a subset of the 912 daughters that were phenotyped and genotyped for the results presented in Table 2. The solid fat content measures were

collected over 2 lactations. Breeding values were calculated using an animal model similar to that of Johnson et al 2000.

5 The same statistical model was fitted for solid fat content as was for the milk component analysis. The DGAT1 polymorphism has a statistically significant effect (p-value <0.0001) on solid fat content, increasing it by approximately 1% for each addition of the Q allele.

10 This effect was further confirmed in 50 daughters (predominantly Holstein-Friesian) that were farmed at one location and measured for SFC on the same day. The estimated effect for of the DGAT1 polymorphism on SFC was to increase it by approximately 2% per addition of each Q allele. This finding was significant at the five percent threshold level.

15 The genetic standard deviation for SFC is 2.25 (D Johnson personal communication) and thus the effect of DGAT1 is approximately 0.5 of a genetic standard deviation.

4. Relative Transcript Levels of the Splice Variant

20 Real time PCR experiments were conducted using reverse transcribed mRNA isolated from lactating bovine mammary gland(s) (see experimental methods). These experiments revealed that the alternatively spliced transcript as shown on Figure 2b, was approximately 100 fold less abundant than the full length transcript.

METHODS SECTION

In order to identify other polymorphisms within the bovine DGAT1 gene, DNA was isolated from sperm, PCR amplified and then using primers designed from the sequence shown in Figures 2a and 2b and/or the cDNA sequence (SEQ ID NO: 4) direct sequenced on an ABI 3100. The breeds examined were:

- Ayrshire, Angler, Belgian Blue, Blond D'Aquitaine, Brown Swiss, Charolais, Red Devon, Devon, Dexter, Friesian, Guernsey, Belted Galloway, Gelbvieh, Hereford, Jersey, Limousin, Longhorn, Maine Anjou, MRI (Meuse-rhine-yssel), Murray Grey, Piedmontese, Romangola, Sahiwal, Santa Gertrudis, Scottish Highland, Shorthorn, South Devon, Sussex, Swedish Red, Simmental, Wagyu, Welsh Black, Angus, and Zebu.
- All the polymorphisms discovered are listed in Table 1, above.

The majority of the primers are also listed in Figure 2b or contained in the cDNA sequence (SEQ ID NO: 4).

Experimental method for the OLA analysis of four SNP's in DGAT1

PCR amplification of the regions containing the polymorphisms

Protocol for the PCR amplification of exon VIII, intron XII and 3' UTR, the regions containing the four polymorphisms that were initially described in the DGAT1 gene.

Component	For 1 sample	Final concentration
HotStar Qiagen Buffer (10 x)	1.5 µl	0.7 µM
Primer 17F at 100µM	0.07 µl	0.7µM
18R at 10 µM	0.07 µl	0.5 µM
Primer 6F at 100µM	0.05µl	0.5 µM
AW 446985dn1 at 100µM	0.05µl	0.5 µM
Primer InsUp1	0.05µl	0.5 µM
14R2	0.05µl	10%
DMSO	1 µl	300 µM
dNTP 10mM	0.3 µl	0.1 U/µl
HotStarQiagen Taq (CatNr 203205:5U/µl)	0.2 µl	
H2O	1.66 µl	
DNA (5 ng/µl)	5 µl	
Total	10 µl	

Primer sequences are given in the following table as well as the genomic region targeted by them.

SNP targeted	Primer name	Primer sequence	5' base position
Exon VIII SNP (DG 1)	17F	CCTGAGCTTGCCTCTCCCACAGT	6579
	18R	CCAGGAGTCGCCGCAGCAGGAAG	7058
Exon XII SNPs (DG 2 and DG3)	6F	CCGCCATCCAGAACTCCATGAAG	7280
	AW446985 dn1	TAGAACTCGCGGTCTCCAAAC	7605
	InsUp1	TGGCTGTCACTCATCATCGGGCA	8222
3'UTR SNP (DG4)	14R2	TTGCACAGCACTTTATTGACACA	8566

PCR amplification was performed on MJ PTC100 or PTCT200 cyclers using the following steps:

Step	Temperature	Time	Comment
1° Activation of the enzyme	94 °C	12 minutes	One times
2° Denaturation	92 °C	1 minute	Repeat step 2 to 4, 35 times
3° Hybridisation	60°C	1 minute 30 seconds	
4° Elongation	72°C	1 minute 30 seconds	
5° Inactivation of the enzyme	99°C	45 minutes	

5

Oligonucleotide Ligation Assay (OLA)

The oligonucleotides used in the OLA multiplex reaction are given in the table below. The detection of each mutation relies on the use of two fluorescent-labelled oligonucleotide (SNPx_FAM and SNP_x_HEX) and one common 3' and 5' phosphorylated, non-labelled oligonucleotide (SNPx_2P)

10

Locus	Oligo	Sequence	5' base position	Number of spacer phosphoramidites	Size of the ligation product*
DG1	SNP1_FAM	AGC TTT GGC AGG TAA GGC	6813	0	32
	SNP1_HEX	AGC TTT GGC AGG TAA GAA	6813		
	SNP1_2P	GGC CAA CGG GGG AG	6831		
DG2	SNP2_FAM	GCT GGC GGT GAG TGA	7424	3	39
	SNP2_HEX	GCT GGC GGT GAG TGG	7424		
	SNP2_2P	CCT GCT GGG TGG GGA	7439		
DG3	SNP3_FAM	GCT GGG TGG GGA CGC	7442	0	29
	SNP3_HEX	GCT GGG TGG GGA CGT	7442		
	SNP3_P	GTG GGG GCG GGT GG	7457		
DG4	SNP4_FAM	TGC CCC AAC CTG GGT	8388	2	36
	SNP4_HEX	TGC CCC AAC CTG GGC	8388		
	SNP4_2P	GCA GCA GGA GGA GGC	8403		

* The size of the ligation products is the sum of the number of nucleotides of the two ligated oligonucleotides plus 3 bases equivalents per spacer phosphoramidites molecule, present at the 5' end of the common oligonucleotide.

15

For each SNP a mixture of the three oligonucleotides was prepared first, following the dilution guidelines in the table below.

SNP mixture	Oligonucleotide to mix	Quantity	Final concentration
DG1 (oligo. mixture)	SNP1_FAM 10 μ M	10 μ l	1 μ M
	SNP1_HEX 10 μ M	20 μ l	2 μ M
	SNP1_2P 10 μ M	20 μ l	2 μ M
	H ₂ O	50 μ l	
DG2 (oligo. mixture)	SNP2_FAM 10 μ M	10 μ l	1 μ M
	SNP2_HEX 10 μ M	20 μ l	2 μ M
	SNP2_2P 10 μ M	20 μ l	2 μ M
	H ₂ O	50 μ l	
DG3 (oligo. mixture)	SNP3_FAM 10 μ M	10 μ l	1 μ M
	SNP3_HEX 10 μ M	20 μ l	2 μ M
	SNP3_2P 10 μ M	20 μ l	2 μ M
	H ₂ O	50 μ l	
DG4 (oligo. mixture)	SNP4_FAM 10 μ M	10 μ l	1 μ M
	SNP4_HEX 10 μ M	30 μ l	3 μ M
	SNP4_2P 10 μ M	20 μ l	2 μ M
	H ₂ O	40 μ l	

The ligation reaction for one sample was performed as follow:

Component	Quantity persample
DG1 oligonucleotide mixture ^a (35, 70 and 70 nM)	0.7 μ l
DG2 oligonucleotide mixture ^a (12.5, 25 and 25 nM)	0.25 μ l
	0.25 μ l
DG3 oligonucleotide mixture ^a (12.5, 25 and 25 nM)	0.25 μ l
	2 μ l
	2 μ l
DG4 oligonucleotide mixture ^a (12.5, 37.5 and 25 nM)	
DMSO	1 μ l
Incubation buffer of the Tsc DNA ligase (Roche, Cat Nr 1 939 807 or 1 939 815)	8.55 μ l
Tsc DNA ligase	5 μ l
H ₂ O	
Multiplex PCR (see above)	
Total	20 μ l

5

^a The final concentration of the oligonucleotides in the ligation reaction is given between parenthesis (SNP_x_FAM, SNP_x_HEX and SNP_x_2P respectively)

10 The sample was submitted to the following temperature cycling program in a MJ PTC100 or PTC 200 PCR machine.

Step	Temperature	Time	Comment
1° Initial denaturation step	98 °C	2 minutes	One times
2° Denaturation	94 °C	30 seconds	Repeat step 2 to 3, 30 times
3° Hybridisation and ligation	45°C	3 minutes	
5° Inactivation of the enzyme	99°C	45 minutes	

15 Following the LCR, 20 μ l of H₂O was added to the ligation reaction. To 0.5 μ l of the diluted ligation reaction, either 2 μ l of loading buffer was added, or 2 μ l loading buffer containing TAMRA350 internal line size standard.

The loading buffer was composed as follows: 1 part of blue dextran (50mg/ml)/ EDTA (25mM) and 6 parts of formamide

The TAMRA350 containing loading buffer was composed as follows: 3 parts TAMRA350 (Applied Biosystems 401736 ; 8nM), 10 parts of Blue dextran (50 mg/ml)/EDTA (25 mM) and 60 parts of formamide.

- 5 TAMRA containing samples was placed alternately with TAMRA free samples when loaded onto the sequencing gel, in order to ease the identification of the lanes on the gel image.

- 10 The samples may require further dilution in order to avoid a too intense fluorescent signal on the sequencer. It is also very likely that from one primer batch to another, oligonucleotides concentrations will need adjustment.

- 15 The samples were denatured for 5 minutes at 95°C before loading. The samples were then loaded onto a 6% denaturing acrylamide gel on sequencer ABI 373 or a 4% gel on sequencer ABI 377.

In addition to the OLA assays referred to above, genotyping of the DGAT1 polymorphism was carried out by utilizing two different techniques for detection of PCR products.

Gel-based Genotyping Assay

- 20 Primer sequences 5' to 3', genomic sequence position in brackets:

DGAT1 21: GTAGCTTTGGCAGGTAAGAA (6811)

DGAT1 22: GGGGCGAAGAGGAAGTAGTA (6984)

DGAT1 23: TGGCCCTGATGGTCTACACC (6613)

DGAT1 24B: GGGCAGCTCCCCCGTTGGCCGC (6850)

25

- The final reaction conditions were 1X Gold PCR buffer, 2.5mM MgCl₂ (Applied Biosystems), 200μM each dNTP (Roche), 600nM DGAT1 21 and DGAT1 22, 400nM DGAT1 23 and DGAT1 24B (Invitrogen), 10% dimethylsulphoxide (Sigma), 3μl DNA template and 2.5 units AmpliTaq Gold DNA polymerase (Applied Biosystems) in a total
30 volume of 50μl.

Cycling conditions were a 94°C initial denaturation for 5 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 20 seconds followed by one extension cycle of 72°C for 2 minutes.

35

Primer positions around polymorphism (in bold) on genomic sequence from 6587 to 6986.

```

                    DGAT123   TGGC  CCTGATGGTC TACACC
5  TGCCTCTCCC ACAGTGGGCT CCGTGCTGGC CCTGATGGTC TACACCATCC

TCTTCCTCAA GCTGTTCTCC TACCGGGACG TCAACCTCTG GTGCCGAGAG

10  CGCAGGGCTG GGGCCAAGGC CAAGGCTGGT GAGGGCTGCC TCGGGCTGGG

GCCACTGGGC TGCCACTTGC CTCGGGACCG GCAGGGGCTC GGCTCACCCC
15

                    DGAT1 21 GTAGCT TTGGCAGGTA AGAA
CGACCCGCCC CCTGCCGCTT GCTCGTAGCT TTGGCAGGTA AGAAGGCCAA
                    ←
                                CGCCGGTT

20  CGGGGGAGCT GCCCAGCGCA CCGTGAGCTA CCCCAGACAAC CTGACCTACC
GCCCCCTCGA CGGG DGAT1 24B

GCGGTGAGGA TCCTGCCGGG GGCTGGGGGG ACTGCCCGGC GGCCTGGCCT

25  GCTAGCCCCG CCCTCCCTTC CAGATCTCTA CTACTTCCTC TTCGCCCCCA
                    ←
                                AT GATGAAGGAG AAGCGGGG DGAT1 22

```

30 The Q allele has polymorphic sequence AA and is detected by the DGAT1 21 + 22 primers, producing a band of 174bp. The q allele has polymorphic sequence GC and is detected by the DGAT123 + 24 primers, producing a band of 238bp.

35 The primers DGAT123 and DGAT122 also successfully PCR the DGAT1 gene producing a product of 372bp in all reactions. Therefore, a QQ homozygote would have bands at 372bp and 174bp, a qq homozygote would have bands at 372bp and 238bp and a Qq heterozygote would have all 3 bands at 372bp, 238bp and 174bp.

18µl of PCR product was separated on a 1.2% agarose TAE gel, stained with ethidium bromide and scored independently by two investigators on the basis of the number and size of bands present.

TaqMan Allelic Discrimination Genotyping Assay

- 5 Primer sequences 5' to 3', genomic sequence position in brackets:

DGAT1forAD: TTCTCCTACCGGGACGTCAA (6651)

ReverseNZ: CCGCGGTAGGTCAGGTTGTC (6890)

Probe sequences 5' to 3', genomic sequence position in brackets:

- 10 ForAA (FAM): CGTTGGCCTTCTTA (6838)

DGAT1ADGC (VIC): TTGGCCGCCTTACC (6836)

Both probes use MGB (minor groove binder) as a non-fluorescent quencher.

- 15 The final reaction conditions are 1x Universal PCR Mastermix (Applied Biosystems), 500nM each primer (Invitrogen), 70nM ForAA (FAM) probe, 300nM DGAT1ADGC (VIC) probe (Applied Biosystems) and 2µl of a 1/20 dilution of DNA template in a total volume of 10µl.

- 20 Cycling conditions were 50°C for 2 minutes, 95°C initial denaturation for 10 minutes, then 37 cycles of denaturation at 94°C for 15 seconds, annealing and extension 60°C for 1 minute.

Primer positions around polymorphism (in bold) on genomic sequence from 6587 to 6986.

- 25 TGCCTCTCCC ACAGTGGGCT CCGTGCTGGC CCTGATGGTC TACACCATCC

DGAT1forAD TTCTCC TACCGGGACG TCAA →

TCTTCCTCAA GCTGTTCTCC TACCGGGACG TCAACCTCTG GTGCCGAGAG

- 30

CGCAGGGCTG GGGCCAAGGC CAAGGCTGGT GAGGGCTGCC TCGGGCTGGG

GCCACTGGGC TGCCACTTGC CTCGGGACCG GCAGGGGCTC GGCTCACCCC

- 35

40

ForAA(FAM) A T TCTTCCGGTTGC
 CGACCCGCCC CCTGCCGCTT GCTCGTAGCT TTGGCAGGTA AGAAGGCCAA
DGAT1ADGC (VIC) CCAT TCCGCCGGTT

5 CGGGGGAGCT GCCCAGCGCA CCGTGAGCTA CCCCACAACT CTGACCTACC
 ← CTGTTG GACTGGATGG

GCGGTGAGGA TCCTGCCGGG GGCTGGGGGG ACTGCCCGGC GGCCTGGCCT
 CGCC ReverseNZ

10

GCTAGCCCCG CCCTCCCTTC CAGATCTCTA CTAATTCTC TTCGCCCCCA

A 240bp product is produced in this reaction. When the Q allele (AA) is present the FAM-labelled probe binds and fluoresces at 518nm. When the q allele (GC) is present the VIC-labelled probe binds and fluoresces at 554nm. After cycling is complete, the plate is scanned on the ABI7900 Sequence Detection System, the fluorescence from each well detected, and a scattergraph is drawn. The scattergraph separates out into 3 clumps with Q homozygotes in the upper left hand corner, q homozygotes in the lower right hand corner and Qq heterozygotes in between. Each clump is circled and the software automatically determines the genotype for each sample. On each plate there are controls with 8 wells each of known Q homozygotes, q homozygotes, Qq heterozygotes and no template controls.

Splice Variant Gene Expression

25 To determine the relative gene expression of the splice variants created by insertion/deletion of 66bp around the polymorphic site by alternate exon usage, RNA was extracted from mammary tissue and reverse transcribed using oligodT primer using a first strand cDNA synthesis kit (Invitrogen). Real time PCR to determine relative quantities of each variant was then carried out.

30

Primer sequences 5' to 3', genomic sequence position in brackets:

DGAT1forRT66: TCTCCTACCGGGACGTCAAC (6652)

DGAT1revRT66: GAGATCGCGGTAGGTCAGGTT (6964)

DGAT1forRTless66: GCTGCTTTGGCAGATCTCTACTACTT (6711)

35 DGAT1revRTless66: AAGCGCTTCGGATGCG (7038)

Probe sequences 5' to 3', genomic sequence position in brackets:

DGAT1with66 (FAM): CCGTGAGCTACCC (6857)

DGAT1less66 (VIC): CTTCGCCCCCACCCT (6976)

- 5 Both probes use MGB (minor groove binder) as a non-fluorescent quencher.

Final reaction conditions were 1X Universal PCR Mastermix (Applied Biosystems), 60nM each primer (Invitrogen), 60nM each probe (Applied Biosystems) and 1µl of template cDNA in a total volume of 10µl.

- 10 Cycling conditions were 50°C for 2 minutes, 95°C initial denaturation for 10 minutes, then 37 cycles of denaturation at 94°C for 15 seconds, annealing and extension 60°C for 1 minute.

- 15 Primer positions around 66bp insertion (in italics) on cDNA sequence. The start of the cDNA sequence is equivalent to position 6479 on the genomic sequence, with the last base of the cDNA equivalent to position 7428 of the genomic sequence.

CCGTGGCCTT TCTCCTCGAG TCTATCACTC CAGTGGGCTC CGTGCTGGCC

- 20 DGAT1forRT66 TCTCCT ACCGGGACGT
CTGATGGTCT ACACCATCCT CTTCTCAAG CTGTTCTCCT ACCGGGACGT

CAAC → DGAT1forRTless66 GCTGCTT
CAACCTCTGG TGCCGAGAGC GCAGGGCTGG GGCCAAGGCC AAGGCTGCTT

- 25 TGGCAG DGAT1with66(FAM) C CGTGAGCTAC
TGGCAGGTAA *GAAGGCCAAC* GGGGGAGCTG CCCAGCGCAC CGTGAGCTAC

- CC ATCTCTAC TACTT →
30 CCCGACAACC TGACCTACCG CGATCTCTAC TACTTCCTCT TCGCCCCAC
← TTGG ACTGGATGGC GCTAGAGDGAT1revRT66 CT TCGCCCCAC

- CCTGTGCTAC GAGCTCAACT TCCCCGCTC CCCCCGCATC CGAAAGCGCT
CCT DGAT1less66 (VIC) ← GCGTAG GCTTTCGCGA

- 35 TCCTGCTGCG GCGACTCCTG GAGATGCTGT TCCTCACCCA GCTCCAGGTG
A DGAT1revRTless66

GGGCTGATCC AGCAGTGGAT GGTCCCGGCC ATCCAGAACT CCATGAAGCC

5 CTTCAAGGAC ATGGACTACT CCCGCATCGT GGAGCGCCTC CTGAAGCTGG

This reaction detects the presence of the insertion splice variant by creating a 145bp product which binds the FAM probe only. The deletion splice variant is detected by a 92bp product that binds the VIC probe only.

10

The cDNA for each alternate splice variant was cloned into pGemT (Promega). A dilution series of the same, known amount, of each variant plasmid DNA was used to create a standard curve that established the linearity of the PCR reaction over a range of DNA concentrations. The threshold cycle number of the sample variants was
15 converted back to a DNA amount by linear regression and the amounts of each variant present compared.

The presence of an alternate splice variant raises the possibility of an alternate function that is at this stage unknown.

20

It will be appreciated that it is not intended to limit the invention to the above examples only, many variations, which may readily occur to a person skilled in the art, being possible without departing from the scope thereof as defined in the accompanying claims.

25

INDUSTRIAL APPLICATION

The present invention is directed to a method of genotyping bovine for improved milk production traits. In particular, such traits include increased milk volume and milk
30 protein content and decreased milkfat content and solid fat content. It is anticipated that herds of bovine selected for such a trait will produce milk which will be more easily processed and such milk and products made therefrom may provide health benefits to consumers, as well as producing an increased milk yield.

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63

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Asp Lys Asp Gly Asp Val Asp Val Gly Ser Gly His Trp Asp Leu Arg
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Cys His Arg Leu Gln Asp Ser Leu Phe Ser Ser Asp Ser Gly Phe Ser
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Asp Pro Ile Gln Val Val Ser Leu Phe Leu Lys Asp Pro Tyr Ser Trp
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Pro Ala Leu Cys Leu Val Ile Val Ala Asn Ile Phe Ala Val Ala Ala
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Leu Ala Leu Met Val Tyr Thr Ile Leu Phe Leu Lys Leu Phe Ser Tyr
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69

245

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255

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15

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<223> cytosine (C) to thymine (T) substitution polymorphism

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<223> adenine (A)-adenine (A) to guanine(G)-cytosine (C) substitution polymorphism
AA corresponds to the Q allele
GC corresponds to the q allele

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reverse primer

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55

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<222> (688)..(709)

60

<223> Primer DgatADGC (VIC)
TTGGCCGCCTTACC
reverse primer

65

<220>

<221> primer_bind

5 <222> (617)..(636)

<223> Primer DgatforRT66
TCTCCTACCGGGACGTCAAC

10 <220>

<221> primer_bind

15 <222> (739)..(760)

<223> Primer DgatrevRT66
GAGATCGCGGTAGGTCAGGTT
reverse primer

20 <220>

<221> primer_bind

<222> (676)..(767)

25 <223> Primer DgatforRTless66
GCTGCTTTGGCAGATCTCTACTACTT
This primer selectively binds and amplifies a contiguous sequence
characteristic of the splice variant & generated by the deletion
bases 689 to 755 of this sequence.

30 <220>

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<222> (817)..(833)

35 <223> Primer DgatrevRTless66
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40 <220>

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<222> (722)..(734)

45 <223> Primer Dgatwith66 (FAM)
CCGTGAGCTACCC

50 <220>

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<222> (771)..(785)

55 <223> Primer Dgatless66 (VIC)
CTTCGCCCCACCCT

60

65

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 ggggacgcgc cgggtccggga cacagacaag gacggagacg tagacgtggg cagcgccac 180
 10 tgggacctga ggtgtcaccg cctgcaggat tccctgttca gttctgacag tggcttcagc 240
 aactaccgtg gcatcctgaa ttggtgtgtg gtgatgctga tcttaagcaa cgcacggta 300
 15 tttctagaga acctcatcaa gtatggcatc ctggtggacc ccatccaggt ggtgtctctg 360
 ttctgaagg acccctacag ctggccagct ctgtgcctgg tcattgtggc caatatcttt 420
 gccgtggctg cgttccaggt ggagaagcgc ctggccgtgg gagctctgac ggagcaggcg 480
 20 gggctgctgc tgcaagggt caacctggcc accattctct gcttccagc ggccgtggcc 540
 tttctcctcg agtctatcac tccagtggg tccgtgctgg cctgatggt ctacaccatc 600
 25 ctcttctca agctgttctc ctaccgggac gtcaacctct ggtgccgaga gcgcagggct 660
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 40 ttctaccggg actggtgaa ctccgagtcc atcacctact tctggcagaa ctggaacatc 1140
 cctgttcaca agtgggtcat cagacacttc tacaagccca tgctccggcg gggcagcagc 1200
 45 aagtgggag ccaggacggc agtgtttctg gcctccgcct tcttccacga gtacctggtg 1260
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 50 ctggcctgga tagtgggccg cttcttccgc ggcaactacg gcaacgcggc cgtgtggctg 1380
 tcactcatca tcgggcagcc ggtggccgtc ctgatgtacg tccacgacta ctacgtgctc 1440
 aaccgtgagg cgcggcagc cggcacctga gcgcctccag gctggccccc tcgtgggtgt 1500
 55 tggactgctt tgccgcgctg cctgcggctg gactagagcc tgccccaacc tgggcgcagc 1560
 aggaggaggc ctggctggtg gaagctgcct cctggcctcc accaggcctc tgctgaagg 1620
 60 gcttctcct gccaggggag agcaggcccg acgcagttct ggccctggg aggtgcccac 1680
 gctctggaaa ccctacagat ctgcaccaag ggtctgaatg tgtcaataaa ga 1732

 <210> 5
 65 <211> 489

74

<212> PRT

<213> Bos taurus

5

<220>

10 <221> VARIANT

<222> (232)..(232)

15 <223> an amino acid substitution (K -> A) caused by a polymorphism at bases 7224-7225 of the genomic sequence (measured from the adenine residue of the translation start codon). Lysine (K) corresponds to the Q allele, alanine (A) corresponds to the q allele.

20 <400> 5

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1 5 10 15

25

Ser Arg Pro Ser Ile Gln Gly Gly Ser Gly Pro Ala Ala Ala Glu Glu
20 25 30

30

Glu Val Arg Asp Val Gly Ala Gly Gly Asp Ala Pro Val Arg Asp Thr
35 40 45

35

Asp Lys Asp Gly Asp Val Asp Val Gly Ser Gly His Trp Asp Leu Arg
50 55 60

40

Cys His Arg Leu Gln Asp Ser Leu Phe Ser Ser Asp Ser Gly Phe Ser
65 70 75 80

45

Asn Tyr Arg Gly Ile Leu Asn Trp Cys Val Val Met Leu Ile Leu Ser
85 90 95

50

Asn Ala Arg Leu Phe Leu Glu Asn Leu Ile Lys Tyr Gly Ile Leu Val
100 105 110

55

Asp Pro Ile Gln Val Val Ser Leu Phe Leu Lys Asp Pro Tyr Ser Trp
115 120 125

60

Pro Ala Leu Cys Leu Val Ile Val Ala Asn Ile Phe Ala Val Ala Ala
130 135 140

65

Phe Gln Val Glu Lys Arg Leu Ala Val Gly Ala Leu Thr Glu Gln Ala
145 150 155 160

70

Gly Leu Leu Leu His Gly Val Asn Leu Ala Thr Ile Leu Cys Phe Pro
165 170 175

75

Ala Ala Val Ala Phe Leu Leu Glu Ser Ile Thr Pro Val Gly Ser Val
180 185 190

	Leu	Ala	Leu	Met	Val	Tyr	Thr	Ile	Leu	Phe	Leu	Lys	Leu	Phe	Ser	Tyr	
			195					200						205			
5																	
	Arg	Asp	Val	Asn	Leu	Trp	Cys	Arg	Glu	Arg	Arg	Ala	Gly	Ala	Lys	Ala	
		210					215					220					
10																	
	Lys	Ala	Ala	Leu	Ala	Gly	Lys	Lys	Ala	Asn	Gly	Gly	Ala	Ala	Gln	Arg	
	225					230					235					240	
15																	
	Thr	Val	Ser	Tyr	Pro	Asp	Asn	Leu	Thr	Tyr	Arg	Asp	Leu	Tyr	Tyr	Phe	
					245					250					255		
20																	
	Leu	Phe	Ala	Pro	Thr	Leu	Cys	Tyr	Glu	Leu	Asn	Phe	Pro	Arg	Ser	Pro	
				260					265					270			
25																	
	Arg	Ile	Arg	Lys	Arg	Phe	Leu	Leu	Arg	Arg	Leu	Leu	Glu	Met	Leu	Phe	
			275					280					285				
30																	
	Leu	Thr	Gln	Leu	Gln	Val	Gly	Leu	Ile	Gln	Gln	Trp	Met	Val	Pro	Ala	
		290					295					300					
35																	
	Ile	Gln	Asn	Ser	Met	Lys	Pro	Phe	Lys	Asp	Met	Asp	Tyr	Ser	Arg	Ile	
	305					310					315					320	
40																	
	Val	Glu	Arg	Leu	Leu	Lys	Leu	Ala	Val	Pro	Asn	His	Leu	Ile	Trp	Leu	
					325					330					335		
45																	
	Ile	Phe	Phe	Tyr	Trp	Leu	Phe	His	Ser	Cys	Leu	Asn	Ala	Val	Ala	Glu	
				340					345					350			
50																	
	Leu	Met	Gln	Phe	Gly	Asp	Arg	Glu	Phe	Tyr	Arg	Asp	Trp	Trp	Asn	Ser	
			355					360					365				
55																	
	Glu	Ser	Ile	Thr	Tyr	Phe	Trp	Gln	Asn	Trp	Asn	Ile	Pro	Val	His	Lys	
		370					375					380					
60																	
	Trp	Cys	Ile	Arg	His	Phe	Tyr	Lys	Pro	Met	Leu	Arg	Arg	Gly	Ser	Ser	
	385					390					395					400	
65																	
	Lys	Trp	Ala	Ala	Arg	Thr	Ala	Val	Phe	Leu	Ala	Ser	Ala	Phe	Phe	His	
					405					410					415		
70																	
	Glu	Tyr	Leu	Val	Ser	Ile	Pro	Leu	Arg	Met							

76

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 5 Gly Gln Pro Val Ala Val Leu Met Tyr Val His Asp Tyr Tyr Val Leu
 465 470 475 480

 Asn Arg Glu Ala Pro Ala Ala Gly Thr
 485
 10
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 <211> 467
 15 <212> PRT

 <213> Bos taurus
 20
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 1 5 10 15

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 20 25 30
 30
 Glu Val Arg Asp Val Gly Ala Gly Gly Asp Ala Pro Val Arg Asp Thr
 35 40 45
 35 Asp Lys Asp Gly Asp Val Asp Val Gly Ser Gly His Trp Asp Leu Arg
 50 55 60

 40 Cys His Arg Leu Gln Asp Ser Leu Phe Ser Ser Asp Ser Gly Phe Ser
 65 70 75 80

 Asn Tyr Arg Gly Ile Leu Asn Trp Cys Val Val Met Leu Ile Leu Ser
 85 90 95
 45
 Asn Ala Arg Leu Phe Leu Glu Asn Leu Ile Lys Tyr Gly Ile Leu Val
 100 105 110
 50
 Asp Pro Ile Gln Val Val Ser Leu Phe Leu Lys Asp Pro Tyr Ser Trp
 115 120 125
 55
 Pro Ala Leu Cys Leu Val Ile Val Ala Asn Ile Phe Ala Val Ala Ala
 130 135 140

 60 Phe Gln Val Glu Lys Arg Leu Ala Val Gly Ala Leu Thr Glu Gln Ala
 145 150 155 160

 Gly Leu Leu Leu His Gly Val Asn Leu Ala Thr Ile Leu Cys Phe Pro
 165 170 175
 65

77

Ala Ala Val Ala Phe Leu Leu Glu Ser Ile Thr Pro Val Gly Ser Val
180 185 190

5 Leu Ala Leu Met Val Tyr Thr Ile Leu Phe Leu Lys Leu Phe Ser Tyr
195 200 205

10 Arg Asp Val Asn Leu Trp Cys Arg Glu Arg Arg Ala Gly Ala Lys Ala
210 215 220

15 Lys Ala Ala Leu Ala Asp Leu Tyr Tyr Phe Leu Phe Ala Pro Thr Leu
225 230 235 240

Cys Tyr Glu Leu Asn Phe Pro Arg Ser Pro Arg Ile Arg Lys Arg Phe
245 250 255

20 Leu Leu Arg Arg Leu Leu Glu Met Leu Phe Leu Thr Gln Leu Gln Val
260 265 270

25 Gly Leu Ile Gln Gln Trp Met Val Pro Ala Ile Gln Asn Ser Met Lys
275 280 285

30 Pro Phe Lys Asp Met Asp Tyr Ser Arg Ile Val Glu Arg Leu Leu Lys
290 295 300

35 Leu Ala Val Pro Asn His Leu Ile Trp Leu Ile Phe Phe Tyr Trp Leu
305 310 315 320

Phe His Ser Cys Leu Asn Ala Val Ala Glu Leu Met Gln Phe Gly Asp
325 330 335

40 Arg Glu Phe Tyr Arg Asp Trp Trp Asn Ser Glu Ser Ile Thr Tyr Phe
340 345 350

45 Trp Gln Asn Trp Asn Ile Pro Val His Lys Trp Cys Ile Arg His Phe
355 360 365

50 Tyr Lys Pro Met Leu Arg Arg Gly Ser Ser Lys Trp Ala Ala Arg Thr
370 375 380

55 Ala Val Phe Leu Ala Ser Ala Phe Phe His Glu Tyr Leu Val Ser Ile
385 390 395 400

Pro Leu Arg Met Phe Arg Leu Trp Ala Phe Thr Gly Met Met Ala Gln
405 410 415

60 Ile Pro Leu Ala Trp Ile Val Gly Arg Phe Phe Arg Gly Asn Tyr Gly
420 425 430

65 Asn Ala Ala Val Trp Leu Ser Leu Ile Ile Gly Gln Pro Val Ala Val
435 440 445

Leu Met Tyr Val His Asp Tyr Tyr Val Leu Asn Arg Glu Ala Pro Ala
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5

Ala Gly Thr
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10

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<212> DNA

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<213> Bos taurus

20

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<223> thymidine (T) to guanine (G) substitution polymorphism

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12

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<212> DNA

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25 <220>
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30 <223> adenine (A) to guanine (G) substitution polymorphism
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40 <223> adenine (A) to guanine (G) substitution polymorphism
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60 <220>
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<222> (5)..(5)
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	<211> 14	
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40	<220>	
	<221> variation	
45	<222> (7)..(8)	
	<223> adenine (A)-adenine (A) to guanine(G)-cytosine (C) substitution polymorphism	
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	GC corresponds to the q allele	
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	<210> 14	
60	<211> 11	
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65	<213> Bos taurus	

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polymorphism
GG-AC
35
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5 <210> 20
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10 <213> Bos taurus

15 <400> 20
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<210> 21

20 <211> 14
<212> DNA

<213> Bos taurus

25

<400> 21
ttggccgcct tacc 14

30

35

CLAIMS:

1. A method of determining genetic merit of a bovine with respect to milk composition and volume which comprises the step of determining the DGAT1 genotypic state of said bovine.
2. A method as claimed in claim 1, wherein the genotypic state is determined with respect to DNA, mRNA and/or protein obtained from said bovine by direct or indirect methods.
3. A method as claimed in claim 1, wherein the genotypic state is determined by the presence of at least one nucleotide difference from the nucleotide sequence encoding bovine DGAT1 (SEQ ID NO: 1) either by direct or indirect methods.
4. A method as claimed in claim 3, wherein the genotypic state is determined by the presence of one or more polymorphisms of SEQ ID NOs: 7 to 19 either by direct or indirect methods.
5. A method as claimed in claim 4, wherein the genotypic state is determined by detecting the presence of the K232A polymorphism (SEQ ID NO: 13), either by direct or indirect methods.
6. A method of selecting a bovine having a desired DGAT1 genotypic state comprising determining the genotypic state according to any one of claims 1 to 5 and selecting said bovine on the basis of said determination.
7. A bovine selected by the method of claim 6.
8. A method of identifying a bovine which possesses a genotype indicative of altered milk production traits, said method comprising:
 - obtaining a nucleic acid sample from said bovine and identifying a polymorphism selected from the group comprising SEQ ID NOs: 7 to 19 of the bovine DGAT1 gene,
 - wherein the presence of said polymorphism is associated with altered milk production traits.

9. A method as claimed in claim 8, wherein said altered milk production traits comprise an increase in milk volume and/or an increased protein to fat ratio of the milk composition or a decrease in milk volume and/or an increase in fat content of the milk composition.
- 5 10. A method as claimed in claim 8, wherein the polymorphism is K232A (SEQ ID NO: 13), wherein the presence or absence of an alanine is associated with the altered milk production traits as claimed in claim 9.
- 10 11. A method as claimed in claim 8, further comprising the step of amplifying said bovine DGAT1 gene sequence.
12. A method as claimed in claim 11, wherein primers selected from the group consisting of (SEQ ID NOs: 20 and 21) are used in said amplification.
- 15 13. A primer suitable for use in detecting a polymorphism selected from the group comprising SEQ ID NOs: 7 to 19 of the bovine DGAT1 gene, said primer consisting of a nucleotide sequence having about at least 12 contiguous bases of SEQ ID NOs. 1, 3 or 4.
- 20 14. A bovine identified by the method of any one of claims 8 to 12.
15. A bovine as claimed in claim 7 or 14, comprising a bull.
- 25 16. Semen produced by a bovine as claimed in claim 15.
17. A bovine as claimed in claim 7 or 14, comprising a cow.
18. Milk produced by a bovine as claimed in claim 17.
- 30 19. Milk as claimed in claim 18, comprising one or more altered characteristics selected from the group consisting of increased % protein; increased total protein content; increased protein to fat ratio; decreased total fat content; increased fat softness; increase unsaturated fat content; increased or decreased solid fat content; and
- 35 increased or decreased total volume; when compared to milk produced by a non-genotyped bovine.

20. Milk as claimed in claim 18, which has a decreased solid fat content, and increased fat softness and is advantageous for use in dairy product processing plants.
- 5 21. A dairy product made from the milk as claimed in any one of claims 18 to 20.
22. A dairy product made from the milk as claimed in claim 20, wherein said product provides a beneficial health effect.
- 10 23. The use of the DGAT1 gene sequence (SEQ ID NOs: 1, 3 and 4) or a fragment or variant thereof, in the identification of one or more molecular DNA markers useful in genotyping and/or selecting a bovine according to the methods of any one of claims 1 to 6 and 8 to 12.
- 15 24. The use of one or more polymorphism sequences selected from the group consisting of SEQ ID NOs: 7 to 19 in a method of identification and selection of a bovine having at least one of said polymorphisms in its DGAT1 gene.
- 20 25. The use of a probe in the methods of genotyping according to any one of claims 1 to 6 and 8 to 12, wherein the probe is selected from any 5 or more contiguous nucleotides of the DGAT1 sequence of SEQ ID NOs: 1, 3 or 4, which is sufficiently complementary with said nucleic acid sequence so as to bind thereto under stringent conditions.
- 25 26. A kit for genotyping a bovine with respect to milk composition and volume associated with DGAT1, comprising a primer or probe as claimed in claim 13 or 25.
27. An isolated nucleic acid molecule having the sequence of SEQ ID NOs: 1 or 4 with or without one or more polymorphisms selected from the group consisting of SEQ
30 ID Nos: 7 to 19, or a fragment or variant thereof.
28. An isolated nucleic acid molecule comprising a DNA molecule having in whole or in part the nucleotide sequence of SEQ ID NOs: 1, 3 or 4 or which varies from the sequence due to the degeneracy of the genetic code, or a nucleic acid strand capable of
35 hybridising with said nucleic acid molecule under stringent hybridisation conditions.

29. An isolated mRNA transcribed from DNA having a sequence which corresponds to a nucleic acid molecule as claimed in claim 27 or 28.
30. A recombinant cloning vector comprising the DNA molecule of claim 27 or 28.
- 5 31. A prokaryotic or eukaryotic cell containing the cloning vector of claim 30.
32. A transfected cell line which expresses a protein encoded by the nucleic acid molecule of claim 27 or 28.
- 10 33. A diagnostic kit useful in detecting a DNA molecule as claimed in claim 27 or 28, comprising a first and second primer for amplifying the DNA, the primers being complementary to nucleotide sequences of the DNA upstream and downstream, respectively, of a polymorphism in the portion of the DNA encoding DGAT1 which
- 15 results in altered relative milk lipid and protein production and milk volume, wherein at least one of the nucleotide sequences is selected to be from a non-coding region of the DGAT1 gene.
34. A kit as claimed in claim 33, further comprising a third primer complementary
- 20 to a polymorphism selected from the group consisting of of SEQ ID NO: 7 to 19 located on the DGAT1 gene.
35. A process for producing a protein encoded by a DNA molecule of claim 27 or 28, comprising the steps:
- 25 a) preparing a DNA fragment including a nucleotide sequence which encodes the protein;
- b) incorporating the DNA fragment into an expression vector to obtain a recombinant DNA molecule which includes the DNA fragment and is capable of undergoing replication;
- 30 c) transforming a host cell with the recombinant DNA molecule to produce a transformant which can express the protein;
- d) culturing the transformant to produce the protein; and
- e) recovering the protein from resulting cultured mixture.
- 35 36. A purified protein having an amino acid sequence of SEQ ID NOs: 2, 5 or 6 or a fragment or variant thereof having biological activity of DGAT1.

37. An antibody raised against the protein as claimed in claim 36.
38. A method for the modulation of milk production and content in a bovine by modulating the activity of the DGAT1 protein.
- 5 39. A method as claimed in claim 38, comprising administering to the bovine an effective amount of a nucleic acid molecule substantially complementary to at least a portion of mRNA encoding the bovine DGAT1 protein and being of sufficient length to sufficiently reduce expression of said DGAT1.
- 10 40. An isolated antisense nucleic acid molecule for use in a method as claimed in claim 39.
41. A method of modulating milk production and/or milk content in a lactating
15 bovine, including administering to the bovine an effective amount of a nucleic acid molecule having ribozyme activity and a nucleotide sequence substantially complementary to at least a portion of mRNA encoding a bovine DGAT1 and being of sufficient length to bind selectively thereto to sufficiently reduce expression of said DGAT1.
- 20 42. A method of inhibiting the activity of bovine DGAT1 in a lactating bovine so as to modulate milk production and/or milk solids content, comprising administering an effective amount of an antibody to the relevant DGAT1.
- 25 43. A method of modulating milk production and/or milk solids content by raising an autoantibody to a bovine DGAT1 in the bovine.
44. A transgenic animal having cells that contain a transgene, which transgene is introduced into the animal or an ancestor of the animal at a prenatal stage, wherein
30 said transgene comprises a bovine cDNA comprising the nucleotide sequence of SEQ ID NO: 4 or an appropriate variant or subsequence thereof.
45. A transgenic non-human animal wherein said endogenous DGAT1 gene has been knocked out and replaced with a transgene.
- 35 46. A transgenic non-human animal, wherein additional copies of the gene encoding DGAT1 are inserted as a transgene.

47. A transgenic non-human animal as claimed in claim 45 comprising a transgenic bovine having a genome lacking a gene encoding a protein having biological activity of DGAT1.
- 5
48. A transgenic non-human animal comprising a mouse having a genome containing a gene encoding a bovine protein having biological activity of DGAT1.
49. A transgenic bovine having a gene encoding a bovine protein having biological
- 10 activity of bovine DGAT1 and heterologous nucleotide sequence antisense to the gene.
50. A transgenic bovine having a genome which includes additional copies of a gene encoding a protein having biological activity of DGAT1 or copies of a gene encoding a protein having biological activity of DGAT1 under control of a high expression promoter.
- 15

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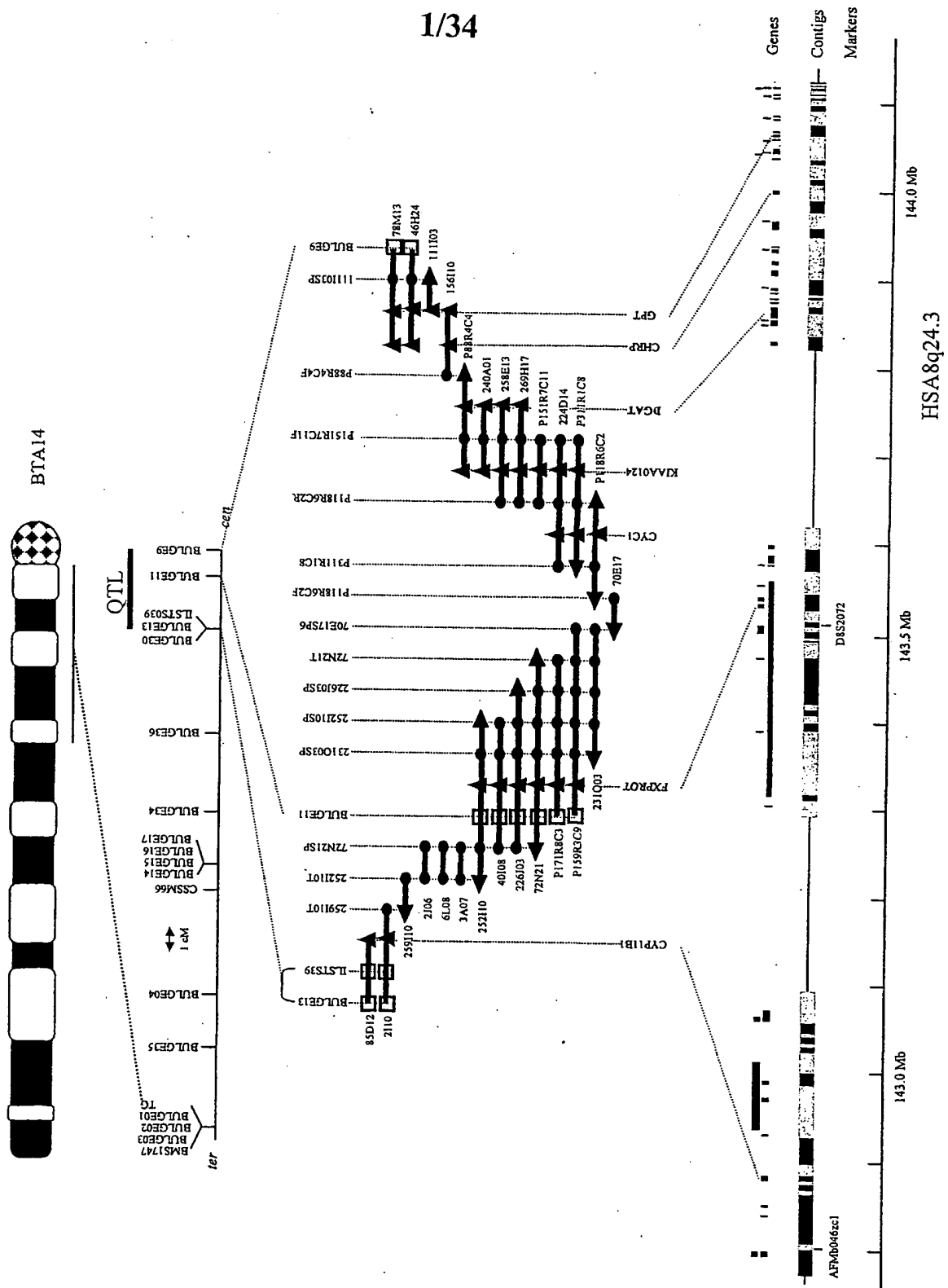


FIGURE 1

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<222> (29)..(31)

<223> bases 1 to 3 of the Kozak recognition sequence. See the genomic sequence from the start codon for bases 4 to 7 of the Kozak recognition sequence or the DGAT1 cDNA for the complete recognition sequence.

acttggccgc gccggggtgc gaactaaggc c

FIGURE 2a

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<210> 1
<211> 11771
<212> DNA
<213> Bos taurus

<220>
<221> CDS
<222> (1)..(191)
<223> Exon 1 CDS, determined by alignment with an amino acid sequence deduced from the cDNA

<220>
<221> misc_signal
<222> (1)..(4)
<223> these bases correspond to bases 4 to 7 of the Kozak recognition sequence. See DGAT1 cDNA for the complete recognition sequence.

<220>
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<222> (3809)..(3896)
<223> Exon 2, determined by alignment with an amino acid sequence deduced from the cDNA

<220>
<221> CDS
<222> (5840)..(5880)
<223> Exon 3, determined by alignment with an amino acid sequence deduced from the cDNA

FIGURE 2b

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<220>

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<222> (5960)..(6045)

<223> Exon 4, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (6138)..(6190)

<223> Exon 5, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (6406)..(6511)

<223> Exon 6, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (6601)..(6714)

<223> Exon 7, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (6815)..(6889)

<223> Exon 8, determined by alignment with an amino acid sequence deduced from the cDNA

FIGURE 2b continued

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<220>

<221> variation

<222> (6824)..(6889)

<223> this sequence is deleted from the alternately spliced transcript.

<220>

<221> CDS

<222> (6960)..(7063)

<223> Exon 9, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (7154)..(7192)

<223> Exon 10, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (7271)..(7312)

<223> Exon 11, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (7386)..(7430)

<223> Exon 12, determined by alignment with an amino acid sequence deduced from the cDNA

FIGURE 2b continued

6/34

<220>

<221> CDS

<222> (7505)..(7617)

<223> Exon 13, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (7705)..(7770)

<223> Exon 14, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (7858)..(7945)

<223> Exon 15, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (8027)..(8089)

<223> Exon 16, determined by alignment with an amino acid sequence deduced from the cDNA

<220>

<221> CDS

<222> (8162)..(8314)

<223> Exon 17 CDS, determined by alignment with an amino acid sequence deduced from the cDNA

FIGURE 2b continued

7/34

<220>

<221> misc_feature

<222> (8315)..(8317)

<223> translation stop codon

<220>

<221> polyA_site

<222> (8572)..(8578)

<223>

<220>

<221> variation

<222> (626)..(626)

<223> thymidine (T) to guanine (G) substitution polymorphism

<220>

<221> variation

<222> (3512)..(3512)

<223> thymidine (T) to guanine (G) substitution polymorphism

<220>

<221> variation

<222> (4040)..(4040)

<223> thymidine (T) to cytosine (G) substitution polymorphism

<220>

<221> variation

<222> (4963)..(4963)

<223> adenine (A) to guanine (G) substitution polymorphism

FIGURE 2b continued

8/34

<220>

<221> variation

<222> (5003)..(5003)

<223> guanine (G) to adenine (A) substitution polymorphism

<220>

<221> variation

<222> (5997)..(5997)

<223> cytosine (C) to thymine (T) substitution polymorphism

<220>

<221> variation

<222> (6829)..(6830)

<223> adenine (A)-adenine (A) to guanine(G)-cytosine (C) substitution polymorphism

AA corresponds to the Q allele

GC corresponds to the q allele

<220>

<221> variation

<222> (6892)..(6892)

<223> guanine (G) to adenine (A) substitution polymorphism

<220>

<221> variation

<222> (7224)..(7225)

<223> guanine (G)-guanine (G) to adenine (A)-cytosine (C) substitution polymorphism

GG-AC

FIGURE 2b continued

9/34

<220>

<221> variation

<222> (7438)..(7438)

<223> adenine (A) to guanine (G) substitution polymorphism

<220>

<221> variation

<222> (7456)..(7456)

<223> cytosine (C) to thymidine (T) substitution polymorphism

<220>

<221> variation

<222> (7987)..(7987)

<223> guanine (G) to adenine (A) substitution polymorphism

<220>

<221> variation

<222> (8402)..(8402)

<223> ctosine (C) to thymidine (T) substitution polymorphism

<220>

<221> misc_feature

<222> (9434)..(9434)

<223> ambiguous nucleotide

<220>

<221> misc_feature

<222> (9496)..(9496)

<223> ambiguous nucleotide

FIGURE 2b continued

10/34

<220>

<221> misc_feature

<222> (10402)..(10417)

<223> ambiguous nucleotides

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<221> primer_bind

<222> (6579)..(6601)

<223> Primer 17F
CCTGAGCTTGCCCTCTCCACAGT

<220>

<221> primer_bind

<222> (7036)..(7058)

<223> Primer 18R
CCAGGAGTCGCCGAGCAGGAAG
reverse primer

<220>

<221> primer_bind

<222> (7280)..(7303)

<223> Primer 6F
CCGGCCATCCAGAACTCCATGAAG

<220>

<221> primer_bind

<222> (7585)..(7605)

<223> Primer AW446985 dn1
TAGAACTCGCGGTCTCCAAAC
reverse primer

FIGURE 2b continued

11/34

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<221> primer_bind
<222> (8222)..(8244)
<223> Primer InsUp1
TGGCTGTCACTCATCATCGGGCA

<220>
<221> primer_bind
<222> (8566)..(8589)
<223> Primer 14R2
TTGCACAGCACTTTATTGACACA

<220>
<221> primer_bind
<222> (6813)..(6830)
<223> Primer SNP1_FAM
AGC TTT GGC AGG TAA GGC

<220>
<221> primer_bind
<222> (6813)..(6830)
<223> Primer SNP1_HEX
AGC TTT GGC AGG TAA GAA

<220>
<221> primer_bind
<222> (6831)..(6844)
<223> Primer SNP1_2P
GGC CAA CGG GGG AG

FIGURE 2b continued

12/34

<220>

<221> primer_bind

<222> (7424)..(7438)

<223> Primer SNP2_FAM
GCT GGC GGT GAG TGA

<220>

<221> primer_bind

<222> (7424)..(7438)

<223> Primer SNP2_HEX
GCT GGC GGT GAG TGG

<220>

<221> primer_bind

<222> (7439)..(7453)

<223> Primer SNP2_2P
CCT GCT GGG TGG GGA

<220>

<221> primer_bind

<222> (7442)..(7456)

<223> Primer SNP3_FAM
GCT GGG TGG GGA CGC

<220>

<221> primer_bind

<222> (7442)..(7456)

<223> Primer SNP3_HEX
GCT GGG TGG GGA CGT

FIGURE 2b continued

13/34

<220>

<221> primer_bind

<222> (7457)..(7470)

<223> Primer SNP3_P
GTG GGG GCG GGT GG

<220>

<221> primer_bind

<222> (8388)..(8402)

<223> Primer SNP4_FAM
TGC CCC AAC CTG GGT

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<221> primer_bind

<222> (8388)..(8402)

<223> Primer SNP4_HEX
TGC CCC AAC CTG GGC

<220>

<221> primer_bind

<222> (8403)..(8417)

<223> Primer SNP4_2P
GCA GCA GGA GGA GGC

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<221> primer_bind

<222> (6811)..(6830)

<223> Primer Dgat 21
GTAGCTTTGGCAGGTAAGAA

FIGURE 2b continued

14/34

<220>

<221> primer_bind

<222> (6965)..(6984)

<223> Primer Dgat 22
GGGGCGAAGAGGAAGTAGTA
reverse primer

<220>

<221> primer_bind

<222> (6613)..(6632)

<223> Primer Dgat 23
TGGCCCTGATGGTCTACACC

<220>

<221> primer_bind

<222> (6829)..(6850)

<223> Primer Dgat 24B
GGGCAGCTCCCCCGTTGGCCGC
reverse primer

<220>

<221> primer_bind

<222> (6651)..(6670)

<223> Primer DgatforAD
TTCTCCTACCGGGACGTCAA

<220>

<221> primer_bind

<222> (6871)..(6890)

<223> Primer ReverseNZ
CCGCGGTAGGTCAGGTGTC
reverse primer

FIGURE 2b continued

15/34

<220>

<221> primer_bind

<222> (6825)..(6838)

<223> Probe ForAA (FAM)
CGTTGGCCTTCTTA

<220>

<221> primer_bind

<222> (6823)..(6836)

<223> Probe DgatADGC (VIC)
TTGGCCGCCTTACC

<220>

<221> primer_bind

<222> (6651)..(6670)

<223> Primer DgatforAD
TTCTCCTACCGGGACGTCAA

<220>

<221> primer_bind

<222> (6878)..(6972)

<223> Primer DgatrevAD
AAGTAGTAGAGATCGCGGTAGGTCA
reverse primer

<220>

<221> primer_bind

<222> (6825)..(6838)

<223> Probe ForAA (FAM)
CGTTGGCCTTCTTA

FIGURE 2b continued

16/34

<220>

<221> primer_bind

<222> (6823)..(6836)

<223> Probe DgatADGC (VIC)
TTGGCCGCCTTACC

<220>

<221> primer_bind

<222> (6652)..(6671)

<223> Primer DgatforRT66
TCTCCTACCGGGACGTCAAC

<220>

<221> primer_bind

<222> (6874)..(6964)

<223> Primer DgatrevRT66
GAGATCGCGGTAGGTCAGGTT
reverse primer

<220>

<221> primer_bind

<222> (6711)..(6972)

<223> Primer DgatforRTless66, GCTGCTTTGGCAGATCTCTACTACTT
This primer was designed to selectively bind and amplify the cDNA
splice variant. The corresponding binding site in this genomic s
equence comprises bases 6711 to 6715, 6815 to 6823 and 6960 to 69
72.

<220>

<221> primer_bind

<222> (7022)..(7038)

<223> Primer DgatrevRTless66
AAGCGCTTTCGGATGCG
reverse primer

FIGURE 2b continued

17/34

<220>

<221> primer_bind

<222> (6857)..(6870)

<223> Probe Dgatwith66 (FAM)
CCGTGAGCTACCC

<220>

<221> primer_bind

<222> (6976)..(6990)

<223> Probe Dgatless66 (VIC)
CTTCGCCCCACCCCT

<400> 1

atg ggc gac cgc ggc ggc gcg ggc ggc tcc cgg cgc cgg agg acg ggg	48
Met Gly Asp Arg Gly Gly Ala Gly Gly Ser Arg Arg Arg Thr Gly	
1 5 10 15	
tcg cgg cct tcg atc cag ggc ggc agt ggg ccc gcg gca gcg gaa gag	96
Ser Arg Pro Ser Ile Gln Gly Gly Ser Gly Pro Ala Ala Ala Glu Glu	
20 25 30	
gag gtg cgg gat gtg ggc gcc gga ggg gac gcg ccg gtc cgg gac aca	144
Glu Val Arg Asp Val Gly Ala Gly Gly Asp Ala Pro Val Arg Asp Thr	
35 40 45	
gac aag gac gga gac gta gac gtg ggc agc ggc cac tgg gac ctg ag	191
Asp Lys Asp Gly Asp Val Asp Val Gly Ser Gly His Trp Asp Leu Arg	
50 55 60	
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tggtggccca ggcctgtcgg cggcagctcg ggctcgagtc cgagagtctg gcgcctggac	311
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ggaggggtctc ctgcccggaa caccaggtta cgggggccga ggggagggca gcggctcaac	431
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caaaggtccc tcatccgtca cccggcgatt gactggccta ggatcctgct tattaccagc	731
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FIGURE 2b continued

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gtacagggga ggagttggaa gtgcgcacac ccacaggtgg gcgccctgct tagctgaagg 851
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 gctatcttca ctgggtgctg tggggagggg agggacaagg gcagttgggg tgcaggcact 1151
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FIGURE 2b continued

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atgaaaccgt cccctgggta tgtggtgcag acatgcacat gcagatgggtg attggcaggt	2771
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ccgtctccgc tctccag g tgt cac cgc ctg cag gat tcc ctg ttc agt tct	3842
Cys His Arg Leu Gln Asp Ser Leu Phe Ser Ser	
65 70 75	
gac agt ggc ttc agc aac tac cgt ggc atc ctg aat tgg tgt gtg gtg	3890
Asp Ser Gly Phe Ser Asn Tyr Arg Gly Ile Leu Asn Trp Cys Val Val	
80 85 90	
atg ctg gtacgtagag tgacaccttg gagcaagggt cctgacggcc ggggggccat	3946
Met Leu	
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ctggcgggtg gggcccttg agcagccgta cctgggacc ctaccgggag catgctcatg	4066
ccgtccctgc tgaatcccag gagatgcctg cagagggcag cctgggagcc tctgagctgg	4126
ggtctgcgcc ccagggggca ctggagtctc cccagggggc gagagagagt aggcagggat	4186

FIGURE 2b continued

20/34

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ggctctggtgg ccctgggtgg gggatggctg ctccgtgggc ccaggccctc cctggcagca 4246
caggtgagtg gtcttggggg tccacgtaga acttcctctt ctgttccaaa ttgccctcat 4306
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tgacagcgtt atgtccctct ctctctatcg cag atc tta agc aac gca cgg tta 5860
                                Ile Leu Ser Asn Ala Arg Leu
                                95                                100

ttt cta gag aac ctc atc aa gtgagtgggc cccggcctgc cccagcccct 5910
Phe Leu Glu Asn Leu Ile Lys
                                105

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FIGURE 2b continued

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gccacctcac ccctcgcccta cacagaccct caccacctg cgtctgcag g tat ggc 5966
Tyr Gly

atc ctg gtg gac ccc atc cag gtg gtg tct ctg ttc ctg aag gac ccc 6014
Ile Leu Val Asp Pro Ile Gln Val Val Ser Leu Phe Leu Lys Asp Pro
110 115 120 125

tac agc tgg cca gct ctg tgc ctg gtc att g gtgagctggg tgcccaggag 6065
Tyr Ser Trp Pro Ala Leu Cys Leu Val Ile
130 135

gcctcaggcc ggcggtgggt gggacagggc tgatctgggc ctgaacctgc cctgggttgc 6125

ttctgtcctc ag tg gcc aat atc ttt gcc gtg gct gcg ttc cag gtg gag 6175
Val Ala Asn Ile Phe Ala Val Ala Ala Phe Gln Val Glu
140 145

aag cgc ctg gcc gtg gtaagcagt ccctcagcc ctcccctgac ttgcctcaag 6230
Lys Arg Leu Ala Val
150

gtccttacca gtcgggctta gggcgggcca ccagctggtc ccaactgtgct tcagggtttt 6290

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gaggtcctct gaccagggag gggggtcctt ggctgacgct ctgctccac ccag gga 6408
Gly

gct ctg acg gag cag gcg ggg ctg ctg ctg cac ggg gtc aac ctg gcc 6456
Ala Leu Thr Glu Gln Ala Gly Leu Leu His Gly Val Asn Leu Ala
155 160 165 170

acc att ctc tgc ttc cca gcg gcc gtg gcc ttt ctc ctc gag tct atc 6504
Thr Ile Leu Cys Phe Pro Ala Ala Val Ala Phe Leu Leu Glu Ser Ile
175 180 185

act cca g gtgggcccc ccccgcccc cggccccgcc cagctgtct cggccacggg 6561
Thr Pro

cagcgcgggg ggcgtggcct gagcttgctt ccccacag tg ggc tcc gtg ctg 6614
Val Gly Ser Val Leu
190

gcc ctg atg gtc tac acc atc ctc ttc ctc aag ctg ttc tcc tac cgg 6662
Ala Leu Met Val Tyr Thr Ile Leu Phe Leu Lys Leu Phe Ser Tyr Arg
195 200 205

gac gtc aac ctc tgg tgc cga gag cgc agg gct ggg gcc aag gcc aag 6710
Asp Val Asn Leu Trp Cys Arg Glu Arg Arg Ala Gly Ala Lys Ala Lys
210 215 220 225

gct g gtgagggtg cctcgggtg gggcactgg gctgccactt gcctcgggac 6764
Ala

cggcaggggc tcggctcacc cccgaccgc cccctgccgc ttgctcgtag ct ttg 6819
Ala Leu

FIGURE 2b continued

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gca ggt aag aag gcc aac ggg gga gct gcc cag cgc acc gtg agc tac Ala Gly Lys Lys Ala Asn Gly Gly Ala Ala Gln Arg Thr Val Ser Tyr 230 235 240	6867
ccc gac aac ctg acc tac cgc g gtgaggatcc tgccgggggc tggggggact Pro Asp Asn Leu Thr Tyr Arg 245 250	6919
gcccggcggc ctggcctgct agccccgccc tcccttcag at ctc tac tac ttc Asp Leu Tyr Tyr Phe 255	6973
ctc ttc gcc ccc acc ctg tgc tac gag ctc aac ttc ccc cgc tcc ccc Leu Phe Ala Pro Thr Leu Cys Tyr Glu Leu Asn Phe Pro Arg Ser Pro 260 265 270	7021
cgc atc cga aag cgc ttc ctg ctg cgg cga ctc ctg gag atg Arg Ile Arg Lys Arg Phe Leu Leu Arg Arg Leu Leu Glu Met 275 280 285	7063
gtgaggcggg gcctcgcggg ccagggtggg cgggcctgcc ggcacccggc accggggctc	7123
agctcactgt ccgcttgctt ccttccccag ctg ttc ctc acc cag ctc cag gtg Leu Phe Leu Thr Gln Leu Gln Val 290	7177
ggg ctg atc cag cag gtacgtgccc gggggggggg gggggggggg gggggggact Gly Leu Ile Gln Gln 295	7232
ctggggccgt tggggagctg actctgcgct ttttgag tgg atg gtc ccg gcc atc Trp Met Val Pro Ala Ile 300 305	7288
cag aac tcc atg aag ccc ttc aag gtgagcaggc aggcctggca gggtaggttc Gln Asn Ser Met Lys Pro Phe Lys 310	7342
cggggtcagg gctgaggag ccagctgtgc cctgtgccca cag gac atg gac tac Asp Met Asp Tyr 315	7397
tcc cgc atc gtg gag cgc ctc ctg aag ctg gcg gtgagtgacc tgctgggtgg Ser Arg Ile Val Glu Arg Leu Leu Lys Leu Ala 320 325	7450
ggacgcgtgg gggcgggtgg ggctgttctg gcacctggca cccactcccc acag gtc Val	7507
ccc aac cac ctc atc tgg ctc atc ttc ttc tac tgg ctc ttc cac tcc Pro Asn His Leu Ile Trp Leu Ile Phe Phe Tyr Trp Leu Phe His Ser 330 335 340 345	7555
tgc ctg aac gcc gtg gct gag ctc atg cag ttt gga gac cgc gag ttc Cys Leu Asn Ala Val Ala Glu Leu Met Gln Phe Gly Asp Arg Glu Phe 350 355 360	7603

FIGURE 2b continued

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tac cgg gac tgg tg gtgggtggcc ttgccggggc ggggggtggtg ggggcccccg 7657
 Tyr Arg Asp Trp Trp
 365

ctggggctgg ggccggagcc cctgcccact ctgccccgcc cccgcag g aac tcc gag 7714
 Asn Ser Glu

tcc atc acc tac ttc tgg cag aac tgg aac atc cct gtt cac aag tgg 7762
 Ser Ile Thr Tyr Phe Trp Gln Asn Trp Asn Ile Pro Val His Lys Trp
 370 375 380 385

tgc atc ag gtgggtgtgc gcctgggggc ggggggttgg ggggtgggac 7810
 Cys Ile Arg

ggggtcgcgt ggccggggcg cccagccac tgccgcctcc cccgcag a cac ttc tac 7867
 His Phe Tyr
 390

aag ccc atg ctc cgg cgg ggc agc agc aag tgg gca gcc agg acg gca 7915
 Lys Pro Met Leu Arg Arg Gly Ser Ser Lys Trp Ala Ala Arg Thr Ala
 395 400 405

gtg ttt ctg gcc tcc gcc ttc ttc cac gag gtcagtgcac tgagggcgcg 7965
 Val Phe Leu Ala Ser Ala Phe Phe His Glu
 410 415

ccctgcccct ggtgggggtg ggggtggggg tgggggctcg ctgacgcccc tctcccctca 8025

g tac ctg gtg agc atc ccc ctg cgc atg ttc cgc ctc tgg gcc ttc acc 8074
 Tyr Leu Val Ser Ile Pro Leu Arg Met Phe Arg Leu Trp Ala Phe Thr
 420 425 430

ggc atg atg gcg cag gtgagcagcc ctggaccccc gctccgcccc gccccgcgag 8129
 Gly Met Met Ala Gln
 435

cgcagaggct cactcccgtc ctgtgtcccc ag atc ccg ctg gcc tgg ata gtg 8182
 Ile Pro Leu Ala Trp Ile Val
 440 445

ggc cgc ttc ttc cgc ggc aac tac ggc aac gcg gcc gtg tgg ctg tca 8230
 Gly Arg Phe Phe Arg Gly Asn Tyr Gly Asn Ala Ala Val Trp Leu Ser
 450 455 460

ctc atc atc ggg cag ccg gtg gcc gtc ctg atg tac gtc cac gac tac 8278
 Leu Ile Ile Gly Gln Pro Val Ala Val Leu Met Tyr Val His Asp Tyr
 465 470 475

tac gtg ctc aac cgt gag gcg ccg gca gcc ggc acc tgagcgctc 8324
 Tyr Val Leu Asn Arg Glu Ala Pro Ala Ala Gly Thr
 480 485

caggctggcc ccctcgtggg tggttgactg ctttgcccg ctgcctgcgg ctggactaga 8384

gcctgccccca acctggggcg agcaggagga ggccctggctg gtggaagctg cctcctggcc 8444

tccaccaggc ctctgcctga agggcttct cctgccaggg gagagcaggc ccgacgcagt 8504

tctggcccct gggaggtgcc catgctctgg aaacctaca gatctcgccc aagggtctga 8564

FIGURE 2b continued

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atgtgtcaat aaagtgtgtg gcacagtgtg ctccctcagc ctccagggca cagggtgtgg 8624
aggagggggc ggccctccca cgtggggcca tgctgtggga aggaggcccc agcgcttga 8684
gaggagctgg ggctgtgtg accctccctg cctcacaggc ctctgtgtg agacgtcttg 8744
ccctgcaagg tggagactcc atgtccaag gccccctgtg cctgaggtct gcacacaagt 8804
ggattcaact tgggtcaggc cagaggctaa ggtgtggaag aaggttgaga atcaggctga 8864
cttgaacggc agcaaagact ccaaggcaag gctgcagaag tctcaaaagc tatgcgaca 8924
gtccctgtct ggggtgtctc cctgggtgtg gctctgggt gcttggaaca agcaggtggc 8984
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tccccgtttt tccaggccct cagcaggtag ttgggtgtgg ccctcatgat accttggtcc 9104
cagagctccg ccaactcaaaa agcttggcag tgaggcaagg gcaaccccgg gctgttcccc 9164
cctctactgg ctctgccgcc tgggttgga accctgaggc tgtgccaggc aggtgtacct 9224
tgacagccag ccatggccca gtaagatggg tgcccagggt ggtacctggg cagcggacct 9284
agctgtgtgt cccccgcccc aaccagaagc cgctctagcc catggtgtgtc gtctgggcga 9344
gacaggtgtg ttggctaggc actgtttgtt ctacagcagg ttaggcagc gtctccctga 9404
cccctgcctt ctaggaagcc accaccctgn gccctactca tcagcaagga cagcgagcag 9464
ggctgagctg ggggtgcgtg ggctgtacg gnccccgcac ctctcatcaca tgcacctctg 9524
caccctctgc tgcctgactc aagagtgggg ggggggggtcc tgtgttctt tcatccaga 9584
cccacggtgc tgaccagtg caccacctg gtccgctagt gctgacctgg ccacagggct 9644
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cgagacactg cgcatcatgc cccctccac cccacccac actggcagga ggaacacaca 9824
gggagaccac acacagagct cgttgtttat aaatctctgc ctggctcatc ggtctgtttg 9884
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ctctctctt ggggcttcta cgagacagtg ggtccttggt ctttggggg ttctgagccc 10184
gtcagcagg agatgggtgg gtcattctct atatttcgtc tccctcgag aagtaggagc 10244
cctccccag ctcgaagagc accggcaggt cgctgtctcc cactccacg gagcccggt 10304
ccaggagcag cagggtgtg gcggtgtagt gcaccagctg ctccctagg ggtgcgactg 10364

FIGURE 2b continued

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ggtcagggtg ccggtggggc cggggggcgg ggtggggnnn nnnnnnnnnn nnncccccc 10424
ccccggcccc agccaccccg cctacgcacg ctggccaggc tgctgtccag gtcgggcagg 10484
ctcatgtcgg gcaccgtaac cgaggggctg aacagctgca gggaagaggg gcgggtcaga 10544
ctgccctgga agcggggcgg gctgggcgcg gggcgggcag ggtagactc acatccagca 10604
gggtgctggt gtccacgctg aagccatggc ttgtcagcat ggtctgcagg ttgtccaggt 10664
tggagtccat ggctccaag tggctcgtga gctcggctct ggccagagga aggggagcag 10724
gtgacgtggc atccaggcac cccaggtgc agtcctgccg gcccttaagc ccagctgcca 10784
gcctgccctg cgagggtca cgggaggccc tgaggtgggg ccctgggcct gggacacttg 10844
gccaccggtg gtggctcagg ctccctcatc accgtgaggc cccgtaaggc agcacgtcag 10904
gctgggccgg ctaagacatc agggggctga gttcagggtc cagaagggcc cggggcccca 10964
gcttctcagg ttacgaacg cctgcccttc aactggagg acagcaggag ggtggagctg 11024
tgctcgcca ggctgggcca gcctaccca gcatgcgcca ggatagatta ggcctgcccc 11084
ctcgaggagg ccaggcagta cctcggggtg ctgcctcagg cccagagggt ggtggggag 11144
cctgttcaca ggctgggag ggtctgtctc agaggctggg gaggggcca agctctggcg 11204
gagccctcct ccctccccag tgggacagcg ctaaccctgg ctggactcgg ccatgcagag 11264
aggaggaggg gcaggggaag aggcgggcga cccagacat ctttgagtg cgagccaaac 11324
tgcaagatag aaagacaaga gccccctct gcgggtgggg ccagcgagt ccaagcccc 11384
caccocgttc acccacctct ggggtggtag gacagacaga ccaggcggcc cggggagggc 11444
ggctcagggg gcaggggtgc ccgccacgtg cctggttccc acggtgcagg ggctgcggga 11504
ccccctggct ggcagtgtct cctccccagc tccatgtctt gggccaggtc ggccagtttc 11564
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tgtcgagggc ccctgcgctt ccagagaccg aagggcgctt ggaaggcact cacttgtcta 11684
ggcaggcgac gctgaggcac ttctcgggag ccgaggcggg gagggtcgac ggatagcggc 11744
cccgagtgat ccgatagaag cttcgta 11771

FIGURE 2b continued

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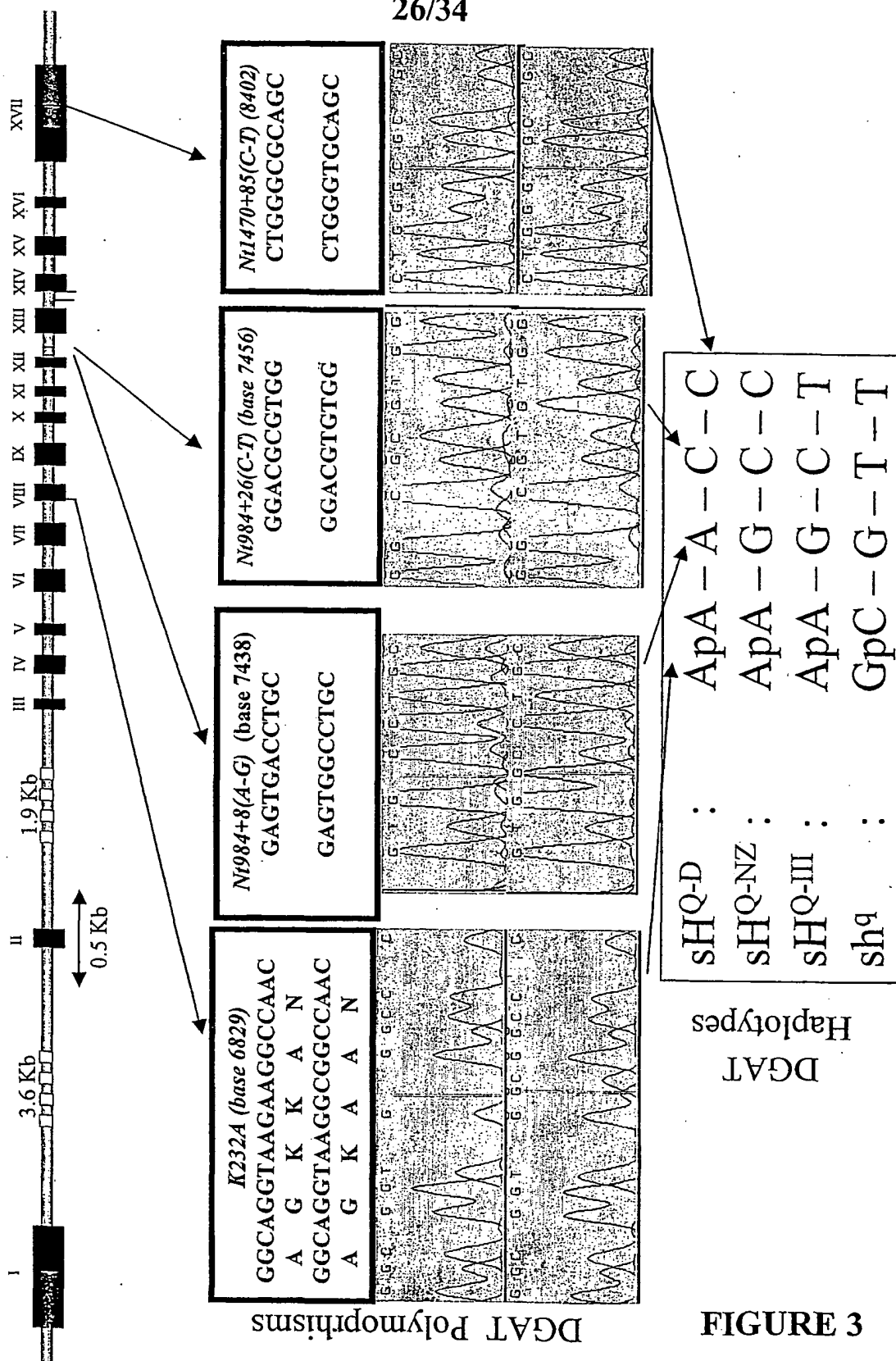


FIGURE 3

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<221> VARIANT

<222> (232)..(232)

<223> an amino acid substitution (K -> A) caused by a polymorphism at bases 7224-7225 of the genomic sequence (measured from the adenine residue of the translation start codon). Lysine (K) corresponds to the Q allele, alanine (A) corresponds to the q allele.

<400> 5

Met Gly Asp Arg Gly Gly Ala Gly Gly Ser Arg Arg Arg Arg Thr Gly
1 5 10 15

Ser Arg Pro Ser Ile Gln Gly Gly Ser Gly Pro Ala Ala Ala Glu Glu
20 25 30

Glu Val Arg Asp Val Gly Ala Gly Gly Asp Ala Pro Val Arg Asp Thr
35 40 45

Asp Lys Asp Gly Asp Val Asp Val Gly Ser Gly His Trp Asp Leu Arg
50 55 60

Cys His Arg Leu Gln Asp Ser Leu Phe Ser Ser Asp Ser Gly Phe Ser
65 70 75 80

Asn Tyr Arg Gly Ile Leu Asn Trp Cys Val Val Met Leu Ile Leu Ser
85 90 95

Asn Ala Arg Leu Phe Leu Glu Asn Leu Ile Lys Tyr Gly Ile Leu Val
100 105 110

Asp Pro Ile Gln Val Val Ser Leu Phe Leu Lys Asp Pro Tyr Ser Trp
115 120 125

Pro Ala Leu Cys Leu Val Ile Val Ala Asn Ile Phe Ala Val Ala Ala
130 135 140

Phe Gln Val Glu Lys Arg Leu Ala Val Gly Ala Leu Thr Glu Gln Ala
145 150 155 160

Gly Leu Leu Leu His Gly Val Asn Leu Ala Thr Ile Leu Cys Phe Pro
165 170 175

FIGURE 4a

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Ala Ala Val Ala Phe Leu Leu Glu Ser Ile Thr Pro Val Gly Ser Val
180 185 190

Leu Ala Leu Met Val Tyr Thr Ile Leu Phe Leu Lys Leu Phe Ser Tyr
195 200 205

Arg Asp Val Asn Leu Trp Cys Arg Glu Arg Arg Ala Gly Ala Lys Ala
210 215 220

Lys Ala Ala Leu Ala Gly Lys Lys Ala Asn Gly Gly Ala Ala Gln Arg
225 230 235 240

Thr Val Ser Tyr Pro Asp Asn Leu Thr Tyr Arg Asp Leu Tyr Tyr Phe
245 250 255

Leu Phe Ala Pro Thr Leu Cys Tyr Glu Leu Asn Phe Pro Arg Ser Pro
260 265 270

Arg Ile Arg Lys Arg Phe Leu Leu Arg Arg Leu Leu Glu Met Leu Phe
275 280 285

Leu Thr Gln Leu Gln Val Gly Leu Ile Gln Gln Trp Met Val Pro Ala
290 295 300

Ile Gln Asn Ser Met Lys Pro Phe Lys Asp Met Asp Tyr Ser Arg Ile
305 310 315 320

Val Glu Arg Leu Leu Lys Leu Ala Val Pro Asn His Leu Ile Trp Leu
325 330 335

Ile Phe Phe Tyr Trp Leu Phe His Ser Cys Leu Asn Ala Val Ala Glu
340 345 350

Leu Met Gln Phe Gly Asp Arg Glu Phe Tyr Arg Asp Trp Trp Asn Ser
355 360 365

Glu Ser Ile Thr Tyr Phe Trp Gln Asn Trp Asn Ile Pro Val His Lys
370 375 380

Trp Cys Ile Arg His Phe Tyr Lys Pro Met Leu Arg Arg Gly Ser Ser
385 390 395 400

Lys Trp Ala Ala Arg Thr Ala Val Phe Leu Ala Ser Ala Phe Phe His
405 410 415

FIGURE 4a continued

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Glu Tyr Leu Val Ser Ile Pro Leu Arg Met Phe Arg Leu Trp Ala Phe
420 425 430

Thr Gly Met Met Ala Gln Ile Pro Leu Ala Trp Ile Val Gly Arg Phe
435 440 445

Phe Arg Gly Asn Tyr Gly Asn Ala Ala Val Trp Leu Ser Leu Ile Ile
450 455 460

Gly Gln Pro Val Ala Val Leu Met Tyr Val His Asp Tyr Tyr Val Leu
465 470 475 480

Asn Arg Glu Ala Pro Ala Ala Gly Thr
485

FIGURE 4a continued

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<400> 6

```

Met Gly Asp Arg Gly Gly Ala Gly Gly Ser Arg Arg Arg Thr Gly
1           5           10           15

Ser Arg Pro Ser Ile Gln Gly Gly Ser Gly Pro Ala Ala Ala Glu Glu
20           25           30

Glu Val Arg Asp Val Gly Ala Gly Gly Asp Ala Pro Val Arg Asp Thr
35           40           45

Asp Lys Asp Gly Asp Val Asp Val Gly Ser Gly His Trp Asp Leu Arg
50           55           60

Cys His Arg Leu Gln Asp Ser Leu Phe Ser Ser Asp Ser Gly Phe Ser
65           70           75           80

Asn Tyr Arg Gly Ile Leu Asn Trp Cys Val Val Met Leu Ile Leu Ser
85           90           95

Asn Ala Arg Leu Phe Leu Glu Asn Leu Ile Lys Tyr Gly Ile Leu Val
100          105          110

Asp Pro Ile Gln Val Val Ser Leu Phe Leu Lys Asp Pro Tyr Ser Trp
115          120          125

Pro Ala Leu Cys Leu Val Ile Val Ala Asn Ile Phe Ala Val Ala Ala
130          135          140

Phe Gln Val Glu Lys Arg Leu Ala Val Gly Ala Leu Thr Glu Gln Ala
145          150          155          160

Gly Leu Leu Leu His Gly Val Asn Leu Ala Thr Ile Leu Cys Phe Pro
165          170          175

Ala Ala Val Ala Phe Leu Leu Glu Ser Ile Thr Pro Val Gly Ser Val
180          185          190

Leu Ala Leu Met Val Tyr Thr Ile Leu Phe Leu Lys Leu Phe Ser Tyr
195          200          205

Arg Asp Val Asn Leu Trp Cys Arg Glu Arg Arg Ala Gly Ala Lys Ala
210          215          220

Lys Ala Ala Leu Ala Asp Leu Tyr Tyr Phe Leu Phe Ala Pro Thr Leu
225          230          235          240

```

FIGURE 4b

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Cys Tyr Glu Leu Asn Phe Pro Arg Ser Pro Arg Ile Arg Lys Arg Phe
 245 250 255
 Leu Leu Arg Arg Leu Leu Glu Met Leu Phe Leu Thr Gln Leu Gln Val
 260 265 270
 Gly Leu Ile Gln Gln Trp Met Val Pro Ala Ile Gln Asn Ser Met Lys
 275 280 285
 Pro Phe Lys Asp Met Asp Tyr Ser Arg Ile Val Glu Arg Leu Leu Lys
 290 295 300
 Leu Ala Val Pro Asn His Leu Ile Trp Leu Ile Phe Phe Tyr Trp Leu
 305 310 315 320
 Phe His Ser Cys Leu Asn Ala Val Ala Glu Leu Met Gln Phe Gly Asp
 325 330 335
 Arg Glu Phe Tyr Arg Asp Trp Trp Asn Ser Glu Ser Ile Thr Tyr Phe
 340 345 350
 Trp Gln Asn Trp Asn Ile Pro Val His Lys Trp Cys Ile Arg His Phe
 355 360 365
 Tyr Lys Pro Met Leu Arg Arg Gly Ser Ser Lys Trp Ala Ala Arg Thr
 370 375 380
 Ala Val Phe Leu Ala Ser Ala Phe Phe His Glu Tyr Leu Val Ser Ile
 385 390 395 400
 Pro Leu Arg Met Phe Arg Leu Trp Ala Phe Thr Gly Met Met Ala Gln
 405 410 415
 Ile Pro Leu Ala Trp Ile Val Gly Arg Phe Phe Arg Gly Asn Tyr Gly
 420 425 430
 Asn Ala Ala Val Trp Leu Ser Leu Ile Ile Gly Gln Pro Val Ala Val
 435 440 445
 Leu Met Tyr Val His Asp Tyr Tyr Val Leu Asn Arg Glu Ala Pro Ala
 450 455 460
 Ala Gly Thr
 465

FIGURE 4b continued

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K232A



Bos taurus:	LALMVYTTILEFLKFSYRDVNLWCRRERRAGAKAKAALAGKKANGGAAQRTVSPDNLTyrDLYYFLFAPTLCY
Bison bison:	LALMVYTTILEFLKFSYRDVNLWCRRERRAGAKAKAALAGKKANGGAAQRTVSPDNLTyrDLYYFLFAPTLCY
Ovis aries:	LALMVYTTILEFLKFSYRDVNLWCRRERRAGAKAKAALAGKKANGGAAQRTVSPDNLTyrDLYYFLFAPTLCY
Sus scrofa:	LALMVYAILFLKFSYRDVNLWCRRERRATAKAKAASAGKKANGGAAQHSVSPDNLTyrDLYYFLFAPTLCY
Homo sapiens:	LALMAHTILEFLKFSYRDVNSWC---RR--ARAKAASAGKKASSAAAPHTVSPDNLTyrDLYYFLFAPTLCY
Cercopithecus aethiops:	LALMVHTILEFLKFSYRDVNLWC---RR--ARAKAASAGKKASSAAAPHTVSPDNLTyrDLYYFLFAPTLCY
M. musculus domesticus:	FALASYSIMFLKLYSYRDVNLWCRR--VKAKAVSTGKKVSGAAAQQAQVSPDNLTyrDLYYFLFAPTLCY
Rattus norvegicus:	FALASYSIIFLKLSSYRDVNLWCRR--VKAKAVSAGKKVSGAAAQNTVSPDNLTyrDLYYFLFAPTLCY

FIGURE 5

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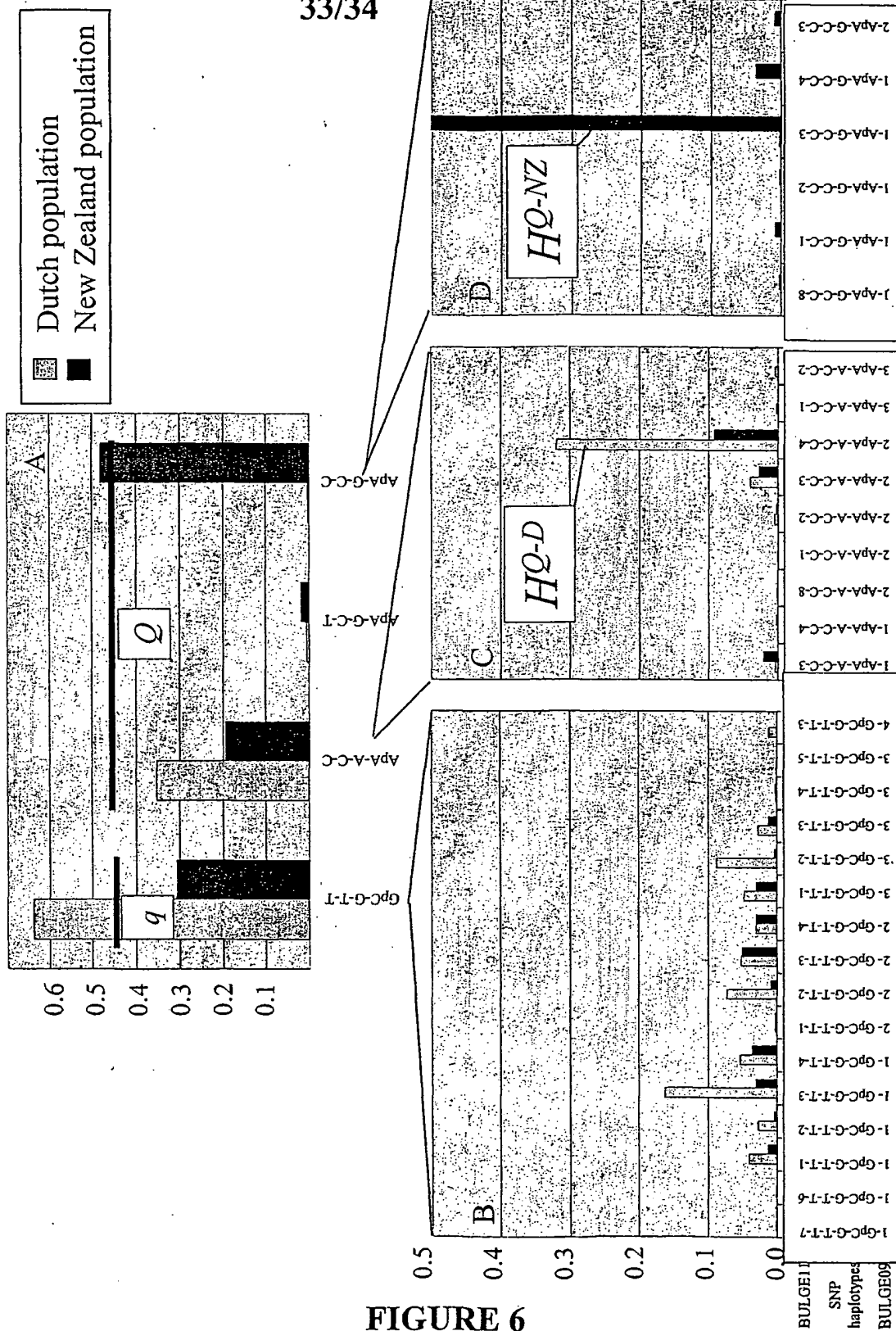


FIGURE 6

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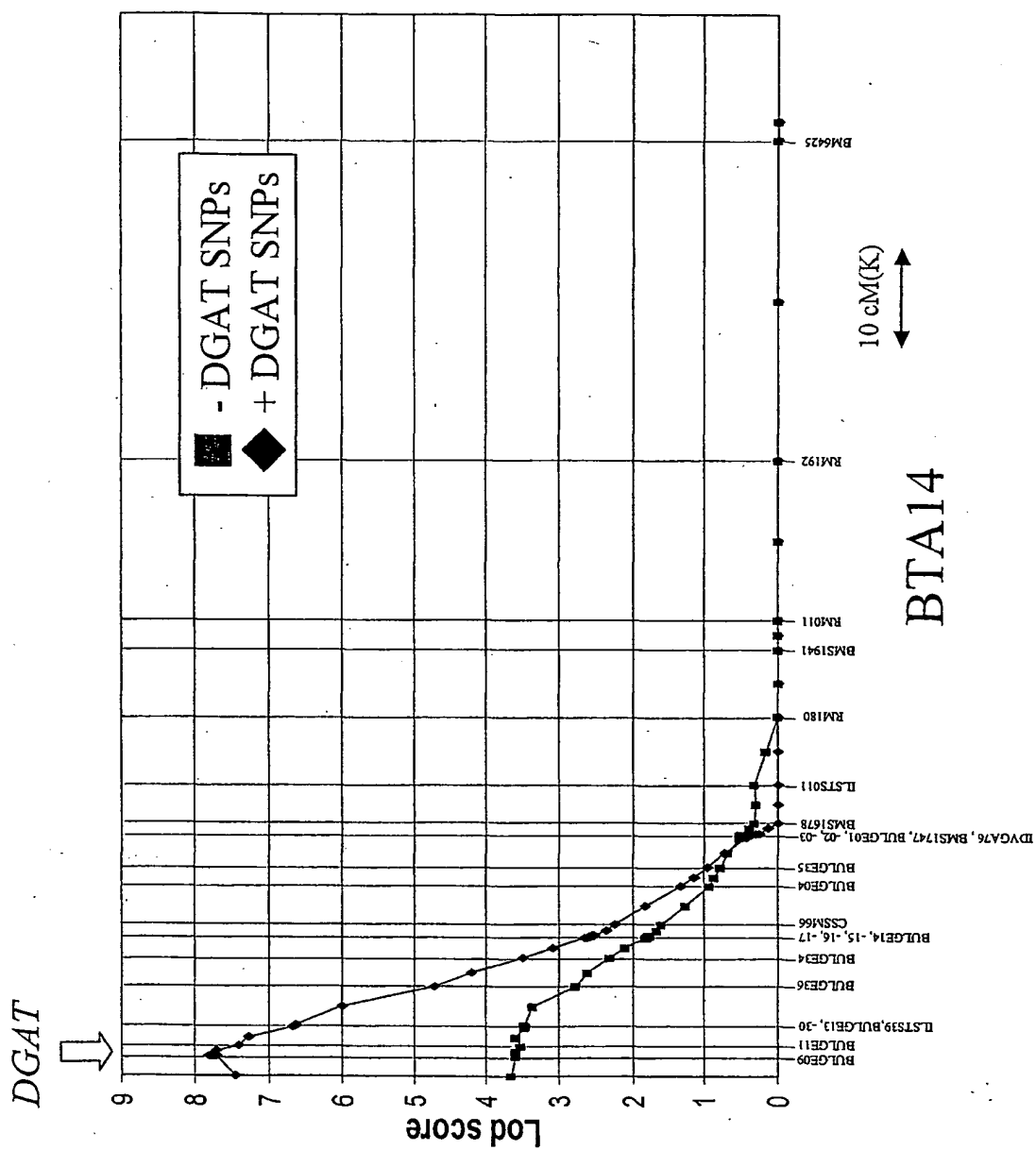


FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ01/00245

A. CLASSIFICATION OF SUBJECT MATTER																						
Int. Cl. ¹ : C12Q 1/68, C12N 15/12, A01K 67/027, C07K 16/40																						
According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols) WPIDS, CA: SEE BELOW																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AGRICOLA, BIOSIS, DNA DATABASES (GENBANK, EMBL): SEE BELOW																						
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPIDS, GENBANK, EMBL, BIOSIS, CA, MEDLINE, AGRICOLA: diacylglycerol acyltransferase, dgat1, bovine, cow, cattle, milk																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
X	EMBL Accession No AF059202 15 October 1998 Oelkers P et al <i>Homo sapiens</i> ACAT related gene product 1 mRNA, complete cds	27-33, 35-37																				
X	EMBL Accession No AF236018 12 August 2000 Joyce CW et al <i>Cercopithecus aethiops</i> diacyl-glycerol acyltransferase mRNA, complete cds	27-33, 35-37																				
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input type="checkbox"/> See patent family annex																						
<p>* Special categories of cited documents:</p> <table border="0"> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier application or patent but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 5 February 2002		Date of mailing of the international search report 13 FEB 2002																				
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer TERRY MOORE Telephone No: (02) 6283 2632																				

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ01/00245

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL Accession No AF078752 11 November 1998 Cases S et al <i>Mus musculus</i> diacylglycerol acyltransferase (Dgat) mRNA, complete cds	27-33, 35-37
X	Smith CJ et al "Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat" (2000) Nature Genetics 25(1), pages 87-90 See the whole document.	44-46

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ01/00245

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos : 1-50 in part and claims 7 and 14-22 in full.
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See the explanation in the supplemental box.
3. ☐ Claims Nos :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ01/00245

Supplemental Box

(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: Box I(2)

Claims 1-50 have not been searched in full because the applicant, although invited to furnish the sequence listing in computer readable form, failed to do so. As such the claims were not searched with regard to the sequences disclosed in the specification. The claims have simply been searched using prior art human and mouse DGAT1 gene sequences located in the EMBL and GENBANK databases.

Claims 7 and 14-22 have not been searched in full because the scope of the claims is unclear. The claims include within their scope bovines that are not the products of the invention and whose pre-existing genotype is simply further characterised using the DGAT1 gene. It is impossible to determine which bovines would fall within the scope of these claims and as such a meaningful search cannot be conducted.

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